

Fig. 2. The effect of plasma from sheep foetuses and from pregnant sheep on the percentage of reticulocytes of the test rabbits. Upper curve: the mean deviations from the initial value on the 4 first days after the injection of foetal sheep plasma; lower curve: the daily mean deviations after the injection of pregnant sheep plasma. The mean errors of the means are drawn as circles. Note the small circles representing the mean errors on the 2nd and 4th day after the injection of pregnant sheep plasma.

In fig. 2 a similar comparison between the variations in the percentage of reticulocytes in the group injected with the plasma from foetuses and from the pregnant sheep is shown. The difference between the two curves may be regarded as statistically significant on the 2nd and 4th day; on the 3rd day no reticulocyte counts were performed in the group which had injections of pregnant sheep plasma.

Summing up, it may be said that foreign plasma, both foetal and adult, tends to call forth a rise in the number of red cells and the reticulocyte percentage in an immunized rabbit. The erythrocytosis, caused by adult plasma, however, is soon replaced by an erythropenia, while after the injection of foetal plasma the erythrocytosis and reticulocytosis are stronger and last for a longer period.

The present material is far too small to allow any valuable conclusions to be drawn as to the changes in the erythropoietic activity of the plasma with increasing foetal age. It might, however, be of some interest to compare the polycythaemic reactions caused by the injection of plasma from different foetuses. Such a comparison is illustrated in fig. 3. As the average red cell values in the test rabbits treated with foetal plasma were above the initial level only on the 1st and 2nd day following the injection (fig. 1), only the values for these 2 first days for each rabbit are compared. It is seen, that the plasma from a foetus aged 71 days caused only a slight increase in the number of red cells. The reac-



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## REDACTORES

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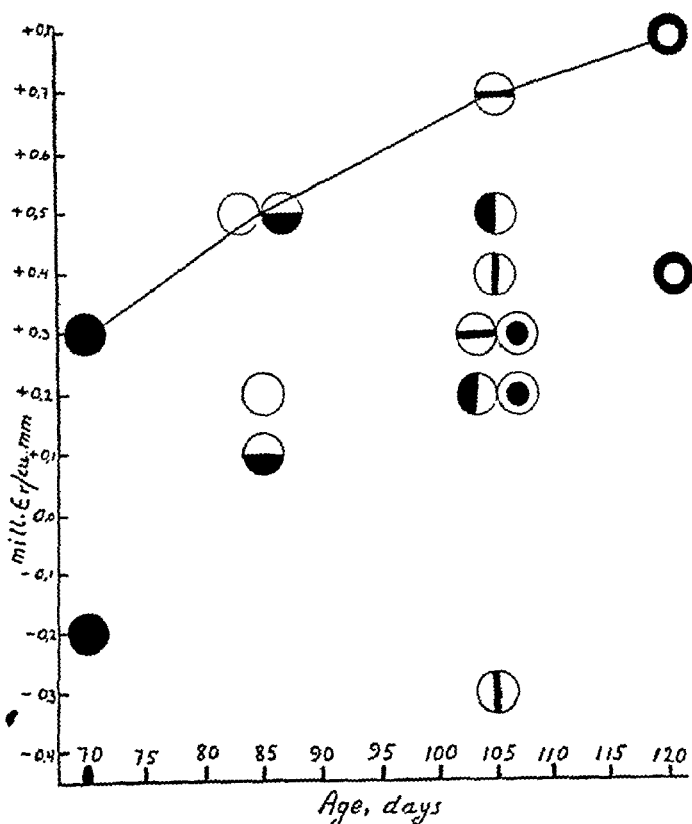


Fig. 3. The effect of plasma from sheep fetuses of different ages on the red cell count of the test rabbits. For each rabbit the deviation from the initial value on the 1st and 2nd day after the injection is considered. To distinguish between the values of different rabbits a different mark for each rabbit is used. A curve is drawn through the points representing the maximal increase of red cells in each age-group.

tion, as may be noted, does not differ from that caused by adult sheep plasma, and hardly exceeds the daily variations met with in untreated rabbits. The 2 rabbits injected with plasma from an 85-day-old foetus showed in the 2nd day an increase of 0.5 mill. in the red cell count, which can hardly be regarded as a random variation. The maximal increases in the rabbits injected with plasma from 105—106 and 121 days old fetuses lie even higher, and exceed by far the normal daily variations. Accordingly a curve drawn through the maximum point for each age group shows a rising tendency. Fig. 3 might be interpreted as showing that while the erythrocytosis-promoting effect of plasma from a 71-day-old foetus is doubtful there seems to be no doubt as to the effect of the plasma from older fetuses.

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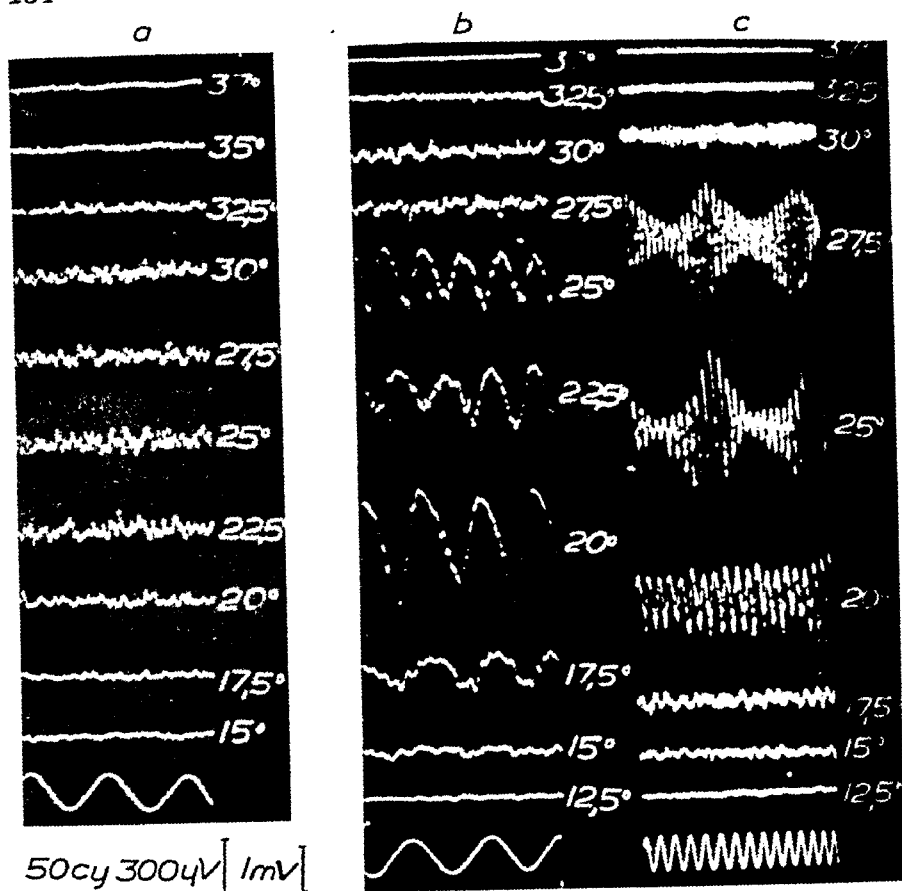


Fig. 5. Motor S 1 root, cat. At beginning of the experiment no discharge to cooling. a. After 10 minutes treatment with  $\text{Ca}^{++}$ -free Krebs solution. b and c. The same root after 5 minutes treatment with Krebs solution free from  $\text{Ca}^{++}$  and  $\text{K}^{+}$ . Higher amplification in a.

tion has been more or less evident in most experiments of this kind. The rhythms never appeared after treatment with Ca-free Krebs alone. It was likewise impossible to produce the rhythmic discharge in a normal nerve, *i. e.* one that had not been treated with Ca-free Krebs, despite the fact that this gave a good discharge to cold.

Since the question of interaction between fibres is of importance and rhythmic discharges are a fundamental property of nerve, these observations were extended to spontaneously active nerve. In order to examine if spontaneously discharging roots were influenced by potassium-free Krebs in the same manner as the discharge to cooling we made experiments with roots treated with sodium phosphate 0.03 M (mixture of primary and secondary

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volleys waxes and wanes with maxima every 30 msec. Fig. 6 c is taken from another experiment and the sweep speed is higher so as to illustrate the two rhythms. The record being monophasic it illustrates the fast and the slow rhythm better than fig. 6 b. In these experiments the discharge and the synchronization, contrary to what is the case with the discharge to cooling, does not depend upon the change of temperature but on the absolute temperature reached. Increase of the concentration of phosphate ions in Krebs solution up to exchange of all sodium chloride (0.154 M) may lead to some synchronization of the discharge but very small in comparison with that seen in fig. 6 after withdrawal of potassium.

It must be emphasized that the rhythms are the same in every experiment, the frequency merely varying with the temperature. The rhythms are therefore not likely to be due to interference between two groups of fibres spontaneously active at different frequencies. This conclusion is strongly supported by fig. 6 c where the small *monophasic* discharges are clearly still synchronized with the 200/sec. frequency but are simply smaller in amplitude. The rhythms seen in the nerves treated with phosphate and in those treated with Ca-free Krebs represent no doubt the same phenomenon and only differing because of the temperature levels at which they appear.

That the two rhythms represent different processes is clear from the fact that they have different temperature coefficients.  $Q_{10}$  of the slow fluctuation is about 10, but of the fast about 2.

It is well known that in citrate- and oxalate-treated nerves the membrane has a tendency to oscillate in a fast rhythm and that impulses may be initiated on the crest of the negative fluctuations (see BRINK and BRONK, 1941, ARVANITAKI 1942, MONNIER 1946, LORENTE DE NÓ 1947). The fact that in our experiment the discharge appears in volleys with a frequency of about 200/sec. must be due to a synchronized excitation of this kind in the region where the impulses are generated.

With regard to the slow oscillations in the height of the responses, since these are recorded in a region of nerve where the environment is normal, it is unlikely that they represent changes in the height of single fibre potentials. It is more likely that they indicate the number of fibres which are excited at each moment by the fast rhythmic process. Now the nerve excitability is known to be closely correlated with the afterpotentials (GASSER

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## Osmotic Pressure Determinations.

Differences in the osmotic pressure of the plasma samples and between the plasma and a 154.0 mM NaCl-solution was determined by means of a slightly modified form — as described by BÁRÁNY (1947) — of Hill-Baldes thermoelectrical vapour pressure method (BALDES et al. 1934, 1939). The determinations were carried out with the thermopiles kept in an air atmosphere. All determinations were made by the same person within a few hours after the drawing of the blood.

The sensitivity of the arrangement, with insignificant variations for the different thermopiles, was 19.9 scale divisions per 1 mM NaCl. The calibration curve was a straight line through the origin. The readings were taken 25 minutes after the piles were placed in the humid chamber. Then the readings remained constant within the error of the method for the reading period. The standard error with respect to a single determination of the difference in osmotic pressure between a 154.0 mM NaCl-solution and a 152.3 mM NaCl-solution was 0.39 mM. The standard error of a single determination of the differences in osmotic pressure between the various plasma samples — calculated from 37 double, 4 triple and 1 quadruple determination — was 0.75 mM NaCl or about 0.5 percent of the total osmotic pressure of the plasma. This error is somewhat greater than that given by BALDES. The accuracy was, however, adequate for the present investigation. The osmotic pressure of the plasma samples was determined by comparing them with the first sample, taken 30 minutes after the end of the urethane injection. This sample was also compared with a 154.0 mM NaCl-solution.

The results are expressed in terms of the millimolarity of the reference solution (154.0 mM NaCl-solution). An osmotic pressure difference of, say, 0.9 mM between two plasma samples thus implies a difference in vapour pressure equal to that between a 154.0 mM and a 153.1 mM NaCl-solution.

## Results.

No significant differences in the osmotic pressure of blood from the salyrgan-treated animals and from the controls were registered. Table 1.

During the experimental period there was a statistically<sup>1</sup> insignificant decrease in the osmotic pressure of the blood. If both groups of animals were treated as one unit the difference was also insignificant, Table 2. In some animals belonging to both groups the difference was, however, so great that it cannot be explained by errors of measurement.

<sup>1</sup> The statistical calculations were made according to the formulas for small samples as given in Fischers Statistical Methods for Research Workers.



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changes that occur in the adipose and other tissues have been studied to a certain extent (DAM and MASON 1945, MASON, DAM and GRANADOS 1946, GRANADOS, MASON and DAM 1947). However, in order to make possible the further study of the relationship between peroxidation and the development of the above mentioned symptoms, it proved fundamental to develop a method for the histochemical demonstration of peroxides. Such a method has been recently developed (GLAVIND, GRANADOS, HARTMANN & DAM 1949), and applied to the study of the relationship between peroxides and the formation of the acid-fast pigment in the adipose tissue of vitamin E deficient rats (GRANADOS, GLAVIND, HARTMANN and DAM to be published).

The works on the influence of dietary fats on the sterility induced by vitamin E deficient diets, and especially those on the rôle played by fats in the bioassay of vitamin E, have been recently reviewed by MASON and HARRIS (1947). Since it has been observed that the quantity and kind of fat are factors of importance in the development of certain symptoms in vitamin E deficiency (DAM 1944 a and b), and that, for instance, 20 % lard added to a vitamin E deficient diet fail to produce the yellow-brown coloration of the adipose tissue induced by 20 % cod liver oil (GRANADOS and DAM 1945 b), we have studied the influence of no dietary fat, 20 % lard, and 20 % cod liver oil on the sterility symptom in vitamin E deficient rats.

### Experimental.

Seventy-eight newly weaned female rats were divided into six groups (13 animals in each), and reared on the diets presented in Table 1. The minimum tocopherol content<sup>1</sup> of the diets given to groups 1, 3 and 5, as determined by DAM and coworkers' method (1948), was of 0.1 mg%, 0.6 mg%, and 0.3 mg%, respectively. Water was available ad libitum. During the first 10 weeks all the animals were weighed weekly.

After 10 preliminary weeks 10 females from each group were mated for 5 days (only one female with one male in each cage) with healthy males between 100 and 150 days of age, which had always been kept on the stock colony diet. From the 12th day after mating had begun all the animals were weighed daily, and inspected grossly for the "placental sign". Three females of each group were left unmated, the aver-

<sup>1</sup> The term "minimum tocopherol content" was chosen because the chloroform extraction used in the method does not with certainty remove any tocopherol present in combination with the dietary casein.

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perature. Even if the localization of the various spots in the coordinate system is not quite accurate, the results found are so unequivocal that it can be said with certainty that the cold spots found at a low temperature are more easily induced to react, when the skin temperature is raised and that other — previously refractory spots — are made to react.

It is possible that the temperature of the single cold spot has a bearing on its mode of reaction. The question of what influence heating the skin corresponding to a cold spot has upon its reactivity is examined in 6 test series on 6 different cold spots each of which has been warmed and thereafter cooled 3—4 times. In 10 of 22 experiments a sensation of cold was released by the cooling, but not in the others. No relation can be demonstrated between the appearance or failure of the cold sensation and the preceding heating time or the heating temperature employed. Neither does the time interval passed after a foregoing cold reaction affect following reactions. When a cold sensation was felt, it lasted from 5 seconds to 2 minutes. This perhaps can be taken as an expression of a greatly varying adaptation, but considering the difficulties which often arise in deciding when cold sensation has disappeared, especially due to tingling and numbness of the arm fixed for a longer time, it becomes doubtful how far these differences are an expression of true variations in adaptation.

The experiments show that the application of heat to the skin at a point corresponding to a cold spot is not in itself sufficient to make the spot sensitive to cold. This means that the difference in sensitivity at various skin temperatures is governed by other factors, possibly changes of temperature in larger areas of the skin or the tissue below.

### Discussion.

The influence of the skin temperature on the number of reacting cold spots may explain the lack of agreement among various authors regarding the number of cold spots per square centimetre. As there are areas of the skin, which never react to cold, even at high temperatures, it must be assumed that there are specific receptors for cold. The cause of the occurrence of a larger number of sensitive spots in warm skin has not been clarified. It is possible that the increase in excitability caused by the higher skin temperature is due to an increase in the rate of restitutional proc-

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### Summary.

1) Fibroblasts from embryonic chicken heart tissue have been cultivated on a simple artificial medium containing dialyzed plasma, dialyzed serum, Tyrode solution and fructose-1.6-diphosphate Ca-salt, glutamine, cystine and glycine. One drop per culture of dialyzed embryonal extract was used as clotting agent. By replacement experiments it could be shown that the four low-molecular components were obligate necessary to the growth and maintenance of the cultures. The experiments have been carried out both in Carrel flasks and in hanging drop cultures.

2) Hypoxanthine added to the above medium, increased the growth ratio. Hanging drop cultures on this medium have been transferred 5 times during a period of 10 days without any visible change of morphological *status*.

3) With aid of paper partition chromatography it has been found that reducing sugars (glucose) organic phosphates, especially aminoethanol phosphoric ester, glutamine, cystine, glycine and hypoxanthine are present in dialysate from calf embryo muscle extract, the latter previously known to contain a complete set of low-molecular accessory growth factors for the cultivation of fibroblasts from embryonal chicken heart tissue *in vitro*.

4) The five components used in the synthetic medium: Glucose + a suitable phosphate source, glutamine, cystine, glycine and hypoxanthine, which also exist in extracts from embryonal tissue could be regarded as the main nutritional low-molecular components for the growth and maintenance of fibroblasts from embryonic chicken heart tissue *in vitro* and *in vivo*.

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Table 2.

*Protein, albumin and relative albumin percentages of the same samples of liquid, native serum with an admixture of 5 per cent of glucose, and after spray-drying of the products containing glucose, representing a total of 371 persons.*

Sample	Pooled Serum			Pooled Serum with admixt. of 5 % of glucose			Spray-dried Serum with admixt. of 5 % of glucose			Dates	Number of Persons
	T	A	$\frac{A \times 100}{T}$	T	A	$\frac{A \times 100}{T}$	T	A	$\frac{A \times 100}{T}$		
A .....	7.06	5.15	72.9	6.68	4.87	72.9	49.19	31.67	64.4	22. X. 1948	63
B .....	7.18	4.72	65.6	6.53	4.36	66.8	49.59	33.37	67.3	11. XI. 1948	166
C .....	6.98	4.67	66.9	6.65	4.48	67.3	50.01	34.85	69.7	15. XI. 1938	107
D .....	7.11	4.69	66.0				49.96	33.97	68.0	3. XII. 1948	35
	7.09	4.80	67.2				49.67	33.56	67.5		371

unteered for this purpose. The blood is drawn with the employment of a tourniquet and a wide cannula, the donors being in the recumbent position. After venesection the quantities of blood obtained from the different donors are pooled, after which the serum is separated and frozen. After an admixture of 5 per cent glucose, and sterilization, the serum passes through the spray-drying chamber with an ingress temperature of 60° to 70° C. Owing to the very rapid evaporation the temperature will, however, hardly exceed 30° C, in the course of the drying itself (MARCUSSEN, 1945). The serum protein analyses were made according to the method of HENRIQUES and KLAUSEN (1932), with precipitation of the globulin fraction under standard conditions with semi-saturation with ammonium sulfate and constant pH and total protein concentration (BING, 1936), a method that on indirect comparison with the electrophoretic patterns of the fractions proved to give a very accurate picture of the ratio between the albumin and globulin concentrations (Bock, 1947).

Duplicate or triplicate determinations were made with each serum. An amino-acetic acid solution of known concentration was used as control of the nitrogen analyses. After solution in a quantity of distilled water suitable for the analyses the samples of dry serum examined were dealt with in exactly the same manner as the liquid sera, with regard to their content both of total protein and of albumin.

The results of an examination of four different pooled sera will appear from Table 2, in which the first and second columns comprise the natural pooled serum before, respectively after, admixture of 5 per cent, of glucose. Column 3 shows the corresponding values in the case of the dried product. As may be seen, there are only slight differences between the serum protein percentages of the



## Studies on Local Anesthetics.

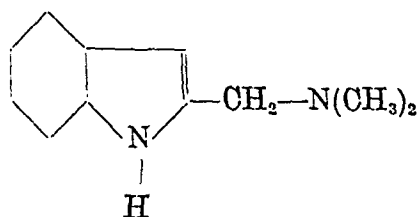
### Pharmacological Properties of Homologues and Isomeres of Xylocain (Alkyl Amino-Acyl Derivatives).

By

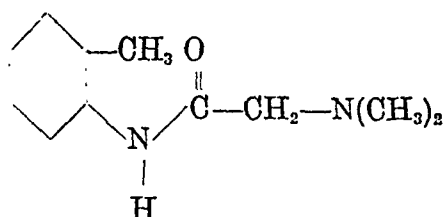
LEONARD GOLDBERG.

Received 2 November 1948.

When synthesizing indol derivatives ERDTMAN and LÖFGREN (1937) found 2-(dimethyl-amino-methyl)-indol (I) to show local anesthetic activity, when rubbed on the lips or on the tongue. An isomere, the alkaloid gramin (II), showed no anesthetic activity, while the  $\omega$ -dimethyl-amino-o-toluidide (III) was found to be anesthetic too. The authors referred the activity to the general formula (IV); in anilides  $R_2$  is substituted by oxygen and in a series of compounds  $R_1$  and  $R_3$  were substituted by hydrogen and  $R_4$  and  $R_5$  by alkyl residues.



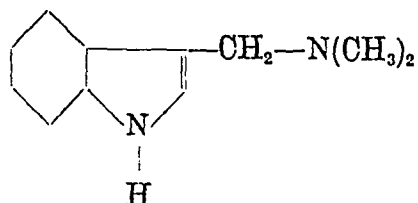
I.



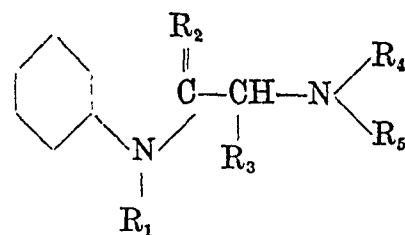
III.

2-(dimethyl-amino-methyl)-indol

$\omega$ -dimethyl-amino-o-toluidide



II.



IV.

gramin (donaxin)

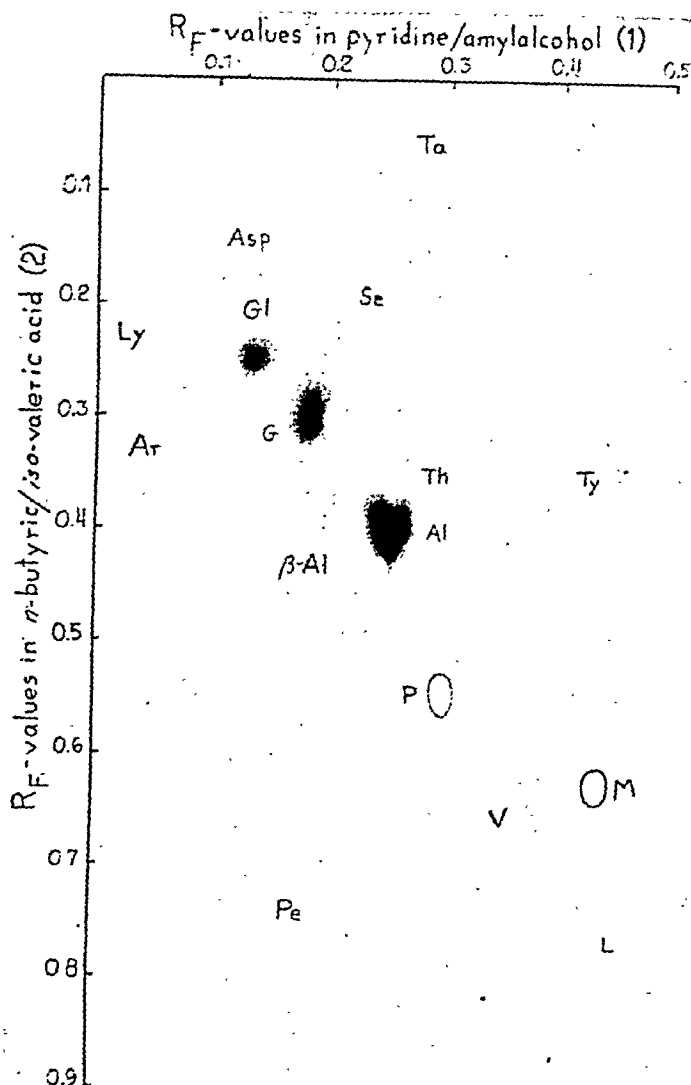


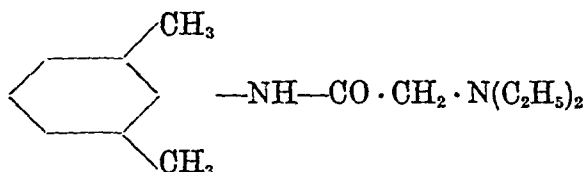
Fig. 1. Photograph of a two-dimensional paper chromatogram showing the distribution of the amino acids from extract of *Calliphora*-pupae, 3 days old. The paper is run using pyridine/amylalcohol (1) and *n*-butyric/iso-valeric acid (2) as solvents. Ta taurine, Asp aspartic acid, Ly lysine, Gl glutaminic acid, Se serine, Ar arginine, G glycine, Th threonine, Al alanine,  $\beta$ -Al  $\beta$ -alanine, Ty tyrosine, P proline, V valine, M methionine, Pe a peptide, L leucine, Il isoleucine. The yellow proline spot is not visible on the photograph. Methionine is not found during this pupal age.

glycine, valine, proline, serine, leucine and iso-leucine, glutaminic acid, aspartic acid, and tyrosine together with a few peptides in low concentrations. Taurine is lacking. No qualitative difference between the sexes was noted. As will be observed the difference

A series of compounds, 16 in all, were prepared, a number of which were tested for their pharmacological properties by U. S. v. EULER. All of these displayed a certain anesthetic activity, tested on the rabbits cornea, and a toxicity about that of procain, but were too irritant to be worth of any further testing or clinical use.

Taking up the concept of anesthetic activity of acetanilide compounds, LÖFGREN and coworkers proceeded and synthesized some ninety compounds, a number of which were alkyl-amino-acyl anilides (LÖFGREN 1946 a, LÖFGREN and LUNDQUIST 1946 b). Other compounds of different chemical constitutions were intended to elucidate the importance of certain chemical characteristics of the anesthetic activity (LÖFGREN and FISCHER 1946 c, LÖFGREN and WIDMARK 1946 d, LÖFGREN 1948).

Epecially one compound,  $\omega$ -diethylamino-2,6-dimethylacetanilide (xylocain, LL 30),



(xylocain, LL 30)  $\omega$ -diethylamino-2,6-dimethylacetanilide

synthesized in 1943, was found by LÖFGREN and LUNDQUIST in experiments on themselves to display promising anesthetic properties. The chemical data of this compound, which was named *xylocain*,<sup>1</sup> were published in 1946 (LÖFGREN 1946 a).

An extensive *pharmacological* study of xylocain in relation to a number of already known local anesthetics, procain, tetracain (pantocain, decicain) and cocain, was begun already in 1943 at this department. A preliminary report was given in 1947 (GOLDBERG 1947 b), showing xylocain to be a potent local anesthetic both for surface and infiltration purposes with a comparatively low toxicity.

A thorough study on the anesthetic activity in teeth of man with due reference to a number of anesthetic parameters was carried out by BJÖRN and HULDT (1947); reports on the use were given by BREMER et al. (1948) in odontology and in surgery by GORDH (1948).

In 1948 LÖFGREN published an extensive study on chemical and physicochemical properties of xylocain and a large body of

<sup>1</sup> Now manufactured by ASTRA, Södertälje, Sweden.

It might be interesting in this connexion to consider the amino acid oxidation during metamorphosis. As appears from Fig. 4, in THUNBERG-experiments an addition to the pupal mass of certain amino acids present in the pupae produces an increase in

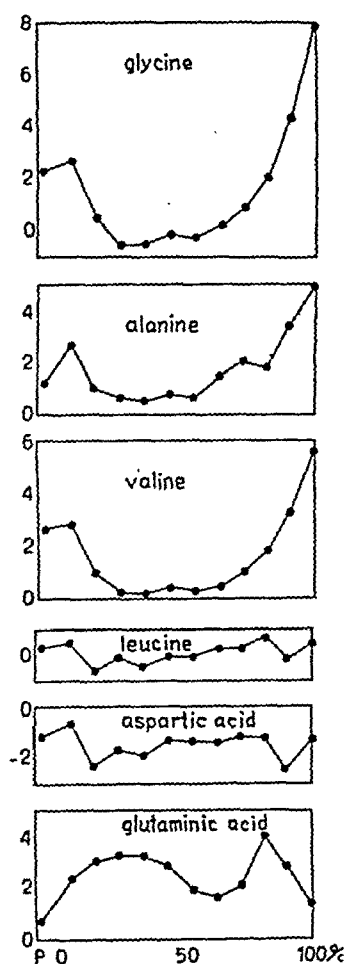


Fig. 4. The change in rate of methylene blue reduction on addition of various amino acids, vertical axis (zero-line = spontaneous decolorization rate) during pupal development of *Calliphora*, horizontal axis.

the Mb-reducing power at the beginning and the end of the metamorphosis period. An addition made in the middle of this period, however, has low effect. Since the concentration of the various amino acids in the pupae at these times is largely the same, Fig. 3, the conclusion might be drawn that the corresponding enzymatic amino acid oxidation should display a U-shaped fluctuation, that is to say it should be high at the beginning and at the end of the metamorphosis period but descend to a minimum between the two. It is interesting that an addition of glycine produces the same variation in the Mb-reduction as an addition of alanine and valine, although glycine is assumed to be oxidized by a different enzyme than the other two amino acids, viz., a special glycine oxidase. A particular study of certain amino acid oxidating enzyme systems verifies the above mentioned assumption. The activity of the apo-enzyme of l-glutaminic dehydrogenase and l-amino acid oxidase varies in such a manner that a minimum occurs in the middle of the pupal period,

Fig. 5. The amino acid oxidation thus displays a fluctuation similar to the total dehydrogenase activity and also to the total metabolism, cf. AGRELL (1947). A high total metabolism involves a high amino acid oxidation and the conversal is also true. The amino acid concentration

in the pupae is, as was previously pointed out, approximatively the same whether the oxidation of these substances is high, as at the beginning and at the end of the pupal period, or at its minimum in the middle of the metamorphosis. This should indi-

related compounds; as a screening test for anesthetic activity the substances were rubbed on the tongue or injected subcutaneously.

The aim of this work is to study a number of isomeres and homologues of xylocain, fifteen in all, from a pharmacological point of view in order to get a concept of the biological effects of this body of alkyl amino-acyl derivatives.

The pharmacological properties studied were convulsive action, toxicity, effect on blood pressure, anesthetic activity on the rabbits cornea and in dermal wheals, compatibility with adrenaline on blood pressure and in local anesthesia, and possible irritant action in the rabbits ear and intra- and subcutaneously in man.

The amounts available of the different compounds were restricted, and only the compounds, seen in table 1, could be chosen for a thorough pharmacological testing.

### Experimental.

The amounts available of the different substances were restricted, and the compounds, seen in table 1, were chosen for this pharmacological study in due collaboration with LÖFGREN at the Dept. of Organic Chemistry of the University of Stockholm, who originally prepared and provided the substances.

Large amounts of xylocain, which was used as a standard, was kindly furnished by ASTRA, Södertälje, Sweden; procain, tetracain and cocain were of the usual commercial brands.

The compounds are denoted by the position of their characteristic radicals: in the benzene nucleus, in the side-chain and at the amino-N.

The compounds were studied in three series:

Series I: Compounds 2, 3, 4, 2,4, 2,6, 2,6  $\alpha$ , 2,4,6, and procain.

Series II: " 2,6, 2,6 $\beta$ , 3,4, 3,5 and procain.

Series III: " 2,6, 2,6nb, 2,6 ib, 2,6 ch and procain.

The compounds of each series were tested simultaneously and compared to procain, compound 2,6 (xylocain) being used as a standard in each series; series I was performed in a more elaborate manner (see below).

The compounds were all prepared as hydrochlorides and were solved as sterile solution 2 % by weight, 0.85 g NaCl added and the acidity adjusted to pH 5.8—5.9; the compounds of series I were also prepared as a 4 % solution by weight, 0.4 g NaCl added pr 100 ml, pH = 5.9.

Compound 2,6 ch was freely soluble only over 40°, and cristallized at room temperature; all others were freely soluble at 18°.

The constitution and molecular weight of the substances are given in table 1 and references are given to chemical data.

Table 2.

The table indicates the number of tests that must be made with 1—9 animals in the group in order to show by the *t*-test that a percentage deviation with different degrees of certainty deviates from 0. The standard deviation of the sample has been supposed to exceed the deviation 0.5, 1, 2 and 4 times. *P* shows the probability that the deviation is due to chance.

	P = 0.1				P = 0.05				P = 0.01				P = 0.001			
Ratio between standard deviation/deviation .....	0.5/1	1/1	2/1	4/1	0.5/1	1/1	2/1	4/1	0.5/1	1/1	2/1	4/1	0.5/1	1/1	2/1	4/1
1 animal/group .....	2.8	4.6	12.8	45.2	3.5	6.4	17.9	63.8	5.2	10.4	30.3	110.2	7.7	16.4	49.3	180.0
2 animals/group .....	2.5	3.5	7.4	23.5	2.9	4.5	10.2	33.2	4.1	7.0	17.2	57.0	6.0	10.7	27.4	93.4
4 animals/group .....	—	2.8	4.6	12.8	—	3.5	6.4	17.9	—	5.2	10.4	30.3	4.9	7.7	16.4	49.3
9 animals/group .....	—	2.4	3.3	6.8	—	2.9	4.3	9.5	—	3.8	6.6	15.6	4.7	5.9	10.0	24.8


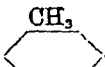
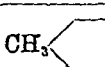
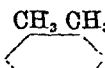
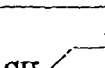
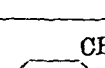
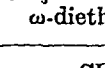
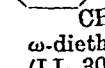
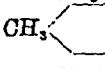
and when *n* is small, these laws are no longer applicable. In such cases recourse must be had to the methods elaborated for small *n*-values, such as the *t*-test and the analysis of variance.

In the *t*-test *t* has a definite value for different degrees of statistical validity and different number of degrees of freedom. According as the statistical validity increases or the number of degrees of freedom diminishes, the *t* value must be increased. If tests are made on several animals simultaneously the variance diminishes and *t* will increase. The number of degrees of freedom can thus be reduced but not so much as  $\frac{N}{a}$  as the effect of reducing the number of degrees of freedom has, relatively speaking, a greater effect on *t* than the diminished variance.

How many tests will have to be made simultaneously on *a* animals relatively to the number of tests on single animals in order to attain the same degree of validity in both cases is indicated by Table 2. The values have been obtained by linear interpolation from the *t*-values given by STUDENT (1925) and by FISHER and YATES (1938) for the degrees of freedom 1—30 and 30—120, respectively. It should be pointed out that, whilst STUDENT indicates the probability that a given positive value will not exceed  $+t$ , FISHER shows the probability that this value,

Table 1.

*Chemical Constitution and Molecular Weight.*

nr	Designation	Chemical Constitution	Mol. weight	Chemical Data <sup>1</sup>
1	2	 $\omega$ -diethylamino-2-methylacetanilide	220.3	O: 8
2	3	 $\omega$ -diethylamino-3-methylacetanilide	220.3	I: 9
3	4	 $\omega$ -diethylamino-4-methylacetanilide	220.3	I: 13
4	2,3	 $\omega$ -diethylamino-2,3-dimethylacetanilide	234.3	I: 11
5	2,4	 $\omega$ -diethylamino-2,4-dimethylacetanilide	234.3	I: 13
6	2,5	 $\omega$ -diethylamino-2,5-dimethylacetanilide	234.3	I: 14
7	2,6	 $\omega$ -diethylamino-2,6-dimethylacetanilide (LL 30, xylocain)	234.3	I: 15
8	3,4	 $\omega$ -diethylamino-3,4-dimethylacetanilide	234.3	I: 17
9	3,5	 $\omega$ -diethylamino-3,5-dimethylacetanilide	234.3	I: 18

(1948) who also denied the existence of an intermediate substance. His concept that "epinephrine is the only sympathetic adrenergic mediator" is, however, in contrast with recent evidence.

It should be noted that EULER used the word "sympathin" in a different sense than did CANNON and ROSENBLUETH. The latter authors used this term for a combination product formed by the mediator from the nerve endings (called M, supposed to be adrenaline) and a specific substance in the effector cell (E and I respectively).

According to EULER (unpublished data) the mesenteric nerves of rabbit contain about 30—40 times more nor-adrenaline than adrenaline. It seems very probable therefore that the substance set free at the nerve endings on stimulation of the mesenteric nerves in the experiments presented here is mainly nor-adrenaline. The effect of electrical stimulation could thus be compared with that obtained after adding nor-adrenaline to the bath. The nor-adrenaline, as well as adrenaline, is believed to act directly upon the sympathetic receptors of the cell, in this case to produce relaxation. As to adrenaline evidence for its direct action upon the receptors of the cell has been produced already by LANGLEY and proven by subsequent authors. The other sympathomimetic amines are also most generally supposed to act directly upon the cell. Some controversial alternatives have been suggested, however. This will be discussed more in detail below.

It seems reasonable to assume that the sympathomimetic amines are capable of reacting with the sympathetic cell receptors and thus produce their sympathomimetic effects because of their chemical relationship to adrenaline and nor-adrenaline. If this be true, it also seems justified to assume that the antagonism studied in these experiments is an example of pharmacological competition. For comparison some results published by UNNA (1943) may be mentioned. He showed that N-allyl-morphine which was less effective than morphine in raising the threshold for pain in mice, could diminish the action of morphine. From his experiments he concluded that N-allyl-morphine by virtue of its chemical relationship to morphine exerted its action upon the same centers as morphine; thus rendering the centers less sensitive to morphine. His results could be considered an example of pharmacological competition as discussed by WOOLLEY (1947).

As illustrated in figure 2 the amines could be divided into three classes with respect to their action upon the intestine.



Table 1. (Cont.)

nr	Designation	Chemical Constitution	Mol. weight	Chemical Data <sup>1</sup>
10	2,4,6	$\begin{array}{c} \text{CH}_3 \\   \\ \text{CH}_3 \text{---} \text{C}_6\text{H}_2 \text{---} \text{NH---CO} \cdot \text{CH}_2 \text{---} \text{N}(\text{C}_2\text{H}_5)_2 \\   \\ \text{CH}_3 \end{array}$ <p><i>ω</i>-diethylamino-2,4,6-trimethylacetanilide (LL 31)</p>	248.4	I: 19
11	2,6 α	$\begin{array}{c} \text{CH}_3 \\   \\ \text{C}_6\text{H}_4 \text{---} \text{NH---CO} \cdot \text{CH} \text{---} \text{N}(\text{C}_2\text{H}_5)_2 \\   \\ \text{CH}_3 \end{array}$ <p><i>α</i>-diethylamino-2,6-dimethylpropionylanilide</p>	248.4	II: 3
12	2,6 β	$\begin{array}{c} \text{CH}_3 \\   \\ \text{C}_6\text{H}_4 \text{---} \text{NH---CO} \cdot \text{CH}_2 \cdot \text{CH}_2 \text{---} \text{N}(\text{C}_2\text{H}_5)_2 \\   \\ \text{CH}_3 \end{array}$ <p><i>β</i>-diethylamino-2,6-dimethylpropionylanilide</p>	248.4	II: 1
13	2,6 nb	$\begin{array}{c} \text{CH}_3 \\   \\ \text{C}_6\text{H}_4 \text{---} \text{NH---CO} \cdot \text{CH}_2 \text{---} \text{NH} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_3 \\   \\ \text{CH}_3 \end{array}$ <p><i>ω</i>-n-butylamino-2,6-dimethylacetanilide</p>	234.4	III: 3
14	2,6 ib	$\begin{array}{c} \text{CH}_3 \\   \\ \text{C}_6\text{H}_4 \text{---} \text{NH---CO} \cdot \text{CH}_2 \text{---} \text{NH} \cdot \text{CH}_2 \cdot \text{CH} \begin{array}{l} \text{CH}_3 \\ \text{CH}_3 \end{array} \\   \\ \text{CH}_3 \end{array}$ <p><i>ω</i>-isobutylamino-2,6-dimethylacetanilide</p>	234.4	III: 4
15	2,6 ch	$\begin{array}{c} \text{CH}_3 \\   \\ \text{C}_6\text{H}_4 \text{---} \text{NH---CO} \cdot \text{CH}_2 \text{---} \text{NH} \cdot \text{CH} \begin{array}{l} \text{CH}_2 \text{---} \text{CH}_2 \\ \text{CH}_2 \text{---} \text{CH}_2 \end{array} \text{CH}_2 \\   \\ \text{CH}_3 \end{array}$ <p><i>ω</i>-cyclohexylamino-2,6-dimethylacetanilide</p>	260.4	IV: 6

<sup>1</sup> 0: 8 denotes substance nr 8 in table 1 in ERDTMAN and LÖFGREN (1937)

I:       "       "       » LÖFGREN (1946 a)  
 II:      "       "       » LÖFGREN and LUNDQUIST (1946 b)  
 III:     "       "       » LÖFGREN and FISCHER (1946 c)  
 IV:      "       "       » LÖFGREN and WIDMARK (1946 d)

### Toxicity.

*Method:* In order to establish the acute toxicity, 2 % solutions of each substance, pH 5.8–5.9, were injected subcutaneously into white mice with a weight of 13–20 g, 5 doses of each concentration to 5 animals in each group; in series I 4 % solutions too were tested according to the same scheme. The doses with the 2 % solution were: 0.30, 0.44, 0.60,

### c. The effects on blood-pressure in vagotomized and sinus denervated animals.

In animals whose vagus nerve has been divided bilaterally before the blasting experiment the arterial blood-pressure drops within the next two or three seconds after the detonation, as in animals which have not been vagotomized. As to the extent of this blood-pressure drop there is no significant difference between vagotomized and non-vagotomized animals. But the blood-pressure appears to remain low for a considerably longer time in vagotomized animals. In surviving, non-vagotomized animals the blood-pressure has usually returned to about the initial value within 4 or 5 minutes. An example may be mentioned here, namely, rabbit K 54 which after bilateral vagotomy had been exposed in the detonation chamber to a maximum pressure of 12.8 kgf/cm<sup>2</sup> and an impulse of 15.1 gf·sec/cm<sup>2</sup>. The blood-pressure fell from an initial value of 110 mm Hg before to 40 mm Hg 12 seconds after the detonation. It remained at between 40 and 55 mm Hg during the next minute, but after that it began to rise again slowly. Ten minutes after the detonation the pressure was 75 mm Hg, and only after approximately 20 minutes had it reached the original value. Large portions of both lungs were haemorrhagic but the haemorrhages were not specially massive.

Also in animals which have been vagotomized and sinus denervated<sup>1</sup> before the blasting, there is a lowering of blood-pressure as in the other animals, though with some differences.

At the moment of the detonation the above-mentioned rise in pressure is seen. This rise, which in the non-treated animals was nearly instantaneous and ceased again very rapidly (duration about 0.02 sec), occurs more slowly here as in the vagotomized animals. Thus, within about 0.1 sec the pressure rises up to a value of 150 to 180 mm Hg. Then within 0.2 to 0.3 sec it falls again to the same, or to a somewhat higher value than before.

After this pressure peak, directly caused by the detonation the arterial pressure is generally somewhat raised for the next 1 or 2 sec, after which it begins to fall rapidly. It drops in a few seconds to very

<sup>1</sup> Denervation of the carotid sinuses was accomplished by ligating and cutting off the internal carotid artery and the tissues between this and the external carotid artery about 0.5 cm above the bifurcation. The completeness of the denervation was checked by letting the animals breathe a mixture of 8 per cent oxygen and nitrogen.

1.00 and 1.5 mg/g body weight, and with the 4 % solution: 0.24, 0.36, 0.54, 0.80 and 1.2 mg/g body weight. The error of the method is approximately 10—15 %.

*Results:* The animals reacted principally in the same way to all substances, the differences being more of a quantitative nature. On a low dose the animals showed no reaction at all or loss of spontaneous activity. On a higher dose the animals showed the Straub tail-phenomenon, the animals moving about, having the tail erected with a slight bow of the tip in a cranial direction. Then convulsions appeared of tonic and clonic type, passing into extension convulsions in the extremities, and opisthotonus: in some cases of a degree to cause the animals to raise on the maximally extended hind limbs and to fall backwards. Then a paralysis developed with loss of righting reflexes, and on higher doses the animals eventually died in respiratory and cardiac failure.

The number of animals in each group showing convulsions, and the number of animals succumbing, were noted. In all 750 animals were used for these experiments.

The median convulsive dose ( $CD_{50}$ ), and the median lethal dose ( $LD_{50}$ ) were computed graphically by plotting the added percentage of animals reacting, transformed to probits according to BLISS (1938), against log dose. The graph also allows of determining the standard deviation, being the inverse value of the declination of the line, representing the dosage effect curve. It was striking to notice, that all substances had approximately the same standard deviation, the difference between them being a shift of the curve, corresponding to the difference in toxicity. The magnitude of the standard deviation, about 40 %, was of the same order as that found for xylocain and procain (GOLDBERG 1947 b).

The values found for the convulsive and lethal doses are tabulated in table 2.

The doses for the 4 % solution (series I) were as a rule lower than those for the 2 % solution, implying the toxicity to increase with increase of concentration (cp. GOLDBERG 1947 b). Here only the values for a 2 % solution will be given.

The lethal doses of the different compounds varied between 0.2 and 2 g/kg, thus a range of 10 times. Extensive tests with xylocain and procain on a large number of animals have shown (GOLDBERG 1947 b) a 2 % solution of xylocain to have a  $LD_{50}$  of 0.6 g/kg and that of procain to be 0.9 g/kg.

From the figures here presented (table 1), the following relation-

*The circulation time is unchanged or shortened in slightly injured animals but prolonged in animals with severe lung injuries.*

## CHAPTER 15.

### Body Temperature in Blast Injury.

No statements are given in the literature as to the body temperature in animals injured by blast.

Also in clinical literature dealing with blast injuries statements as to the body temperature are noticeably sparse. Usually, when first examined after the accident, the injured seem to have had subnormal or normal temperature, which has then risen, particularly if complications have set in. TUNBRIDGE and WILSON (1943) mention one case, a man aged 38, who was injured when a flying bomb detonated at a distance of less than 14 m. One hour after the accident the temperature was 36.1° C. Seven hours later it was 36.9° C, and severe symptoms of blast injury had developed. The following morning the temperature was 37.1° C. On the third day it had risen to 39.7° C probably due to pneumonia having set in. The temperature never fell below 39.3° C, and the man died on the fourteenth day after the accident. In two other cases the temperature was 36.4° C and 37.1° C, respectively, and in a third 36.8° C, 2 hours after the detonation. BARROW and RHOADS (1944) state that on admission to hospital the body temperature in most of their patients injured by blast was slightly subnormal. In those severely injured there was a secondary rise in temperature of 1 or 2° C after 4 to 12 hours.

#### Own investigations.

The body temperature of the rabbit is normally subjected to greater variations than that of man, and seems more dependent on the temperature of the surrounding air. Determinations of the rectal temperature of 40 normal animals gave a mean value of  $38.9 \pm 0.05^\circ \text{C}$  ( $\sigma = 0.29^\circ \text{C}$ ). The variations during two consecutive days are shown in table 34, where the mean values from determinations carried out at three different times of the day have been entered.

As is seen the temperature is remarkably constant during the day. The condition for this, however, is that the animals must be accustomed to the procedure of measurement, that they are absolutely still and calm, and that they are not exposed to draught or strong temperature changes. If the animals are restless the body temperature rises and may increase by 0.5° C, or more. Determinations of the tempera-

Table 2.

*Toxicity and Anesthetic Action.*

Substance <sup>1</sup>	Toxicity			Duration of Anesthetic Action					
				Rabbits Cornea			Dermal wheals (guinea-pig)		
	Convulsive Dose (CD <sub>50</sub> ) g/kg	Lethal Dose LD <sub>50</sub> g/kg	CD <sub>50</sub> /LD <sub>50</sub> in Per Cent	Ab-solute > 5 gf min.	Rel-ative 1 gf min.	Total hrs	1 % min.	2 % min.	2 % + 20 µg/ml adren-alin min.
2 .....	0.3	1.8	20	30	90	5	131	126	247
3 .....	0.4	1.5	25	25	80	5	171	238	353
4 .....	0.3	0.7	43	30	170	6	204	285	319
2,3 .....	0.5	0.8	60	30	250	7	165	220	313
2,4 .....	0.5	0.7	70	40	170	6	208	288	359
2,5 .....	0.5	1.1	45	40	160	5 1/2	228	311	321
2,6 (xyloc.)	0.3	0.4	75	60	180	5 1/2	152	183	332
3,4 .....	0.5	1.2	40	12	140	5	209	222	313
3,5 .....	0.7	2.0	30	18	130	6	221	242	338
2,4,6 .....	0.2	0.2	90	40	180	5 1/2	201	202	272
2,6 α .....	0.3	0.4	75	70	210	6 1/2	238	276	344
2,6 β .....	0.2	0.3	70	60	300	7	150	172	242
2,6 nb.....	0.2	0.3	70	60	220	6	100	155	319
2,6 ib.....	0.2	0.5	40	52	240	6	130	161	320
2,6 ch.....	0.15	0.2	75	43	240	4 1/2	107	190	270
Procain ...	0.4	0.6	70	—	—	—	137	151	237

<sup>1</sup> Cp. table 1.

ship between chemical constitution and lethal effect can be assumed:

a) One methyl group in 2- or 3-position, or two methyl groups in 2,5 and 3,5-position have the lowest lethal effect.

b) A methyl group in 4-position increases the lethal effect, if alone or combined in a 2,4-, in a 3,4- or in a 2,4,6-position, and has about the same effect as a 2,5-position.

c) Two methyl groups in 2,6-position increase the lethal effect.

d) Three methyl groups have the strongest lethal effect (2,4,6).

e) A lengthening of the side-chain increases the lethal effect (2,6 β), whereas a branching does not change it (2,6 α).

f) A n-butyl (2,6 nb) or a cyclo-hexyl radical (2,6 ch) increase the lethal effect as compared to a diethyl radical (2,6) or an iso-butyl radical (2,6 ib).

As to the convulsive action, the doses varied for all compounds between 0.15 and 0.5 g/kg. The determinations are as a rule not so exact as to allow of a definite ranking, the error being 15—25 %.

creased formation of certain organic acids, particularly lactic acid in the blood.

In the slightly injured animals the alkali reserve decreases during the first two hours but after that it rises again quickly above the initial value. This latter increase may be explained as an excess compensation brought about by the hyperventilation.

The increase of non protein nitrogen and the phosphorus content in plasma, usually seen in traumatic shock, appears in blast injury in animals only which have obtained such severe lesions that they die within a short time after the detonation. An increase, though even this only pre-mortal, is observed also in regard to potassium in plasma. The creatinine content does not change.

Thus none of the changes, such as increase of non protein nitrogen and of creatinine, phosphorus and potassium in plasma, which would be attributed to hypoxemia of the kidneys, occur in animals which survive the detonation, and only the reduced alkali reserve indicates a hypoxemia of the muscles (formation of acids).

*The results of the blood analyses thus speak against traumatic shock having been of any importance.*

## CHAPTER 17.

### Causes of Death in Blast Injury.

KROHN *et coll.* (1942) point out that "there are a number of ways of being killed by blast, of which total disintegration of the body is the extreme case met with very close to an explosion". A total disintegration of the body due to blast, however, would be rare, as it only occurs in the immediate vicinity of the charge within the area of the fire ball. As regards the cause of death in the majority of cases, with no external injuries, or only quite insignificant external injuries, there have been many divergent opinions. It has indeed been possible to point to the extensiveness of the lung lesions, but the majority of authors have been inclined to agree with HADFIELD *et coll.* (1940), that the pathologico-anatomic changes are insufficient in causing death. It seems therefore just to declare, that the investigations published so far give no definite reasons for the cause of death in blast injury (cf. also TUNBRIDGE and WILSON 1943).

On the whole there is a certain relationship between convulsive and lethal dose, those with a low toxicity, however, having a relatively high convulsive action (2 and 3),  $CD_{50}$  being a low percentage of  $LD_{50}$ . One limit can be taken at 40—50 % (table 2), compounds below this limit having a strong convulsive and paralyzing action.

The dose for loss of righting reflexes was approximately equal to or slightly higher than the convulsive dose.

### Effect on Blood Pressure.

#### a. Pure compounds.

*Method:* The effect on blood pressure was established in the rabbit. The animal was anesthetized by 1.75 g urethan per kg intravenously in a 20 % solution; the trachea and one jugular vein were cannulated and the arterial blood pressure was recorded from one carotid artery by means of rubber tubings and Hg manometer. The compound was injected into the jugular vein in a 2 % solution, pH 5.8—5.9, together with 3—5 ml of Ringer solution. Several compounds were compared in the same animal. In all some 400 injections (a and b inclusive), were made and evaluated in 15 animals.

*Results:* All substances had a depressant action on the rabbit's blood pressure, and gave an average fall in pressure of about 20—30 mm Hg on a dose of 1.3—2 mg/kg. The action was unaffected by atropin or cutting of the vagi. A typical record is shown in figure 1.

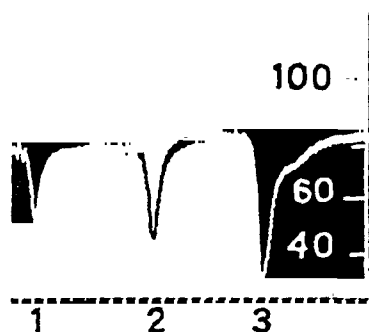


Fig. 1. Effect of Compounds nrs 4 and 2,6  $\alpha$  on Arterial Blood Pressure. Rabbit, 3.0 kg. Time: 30 seconds. 2 % Solutions.

1. 0.25 ml nr. 4. 2. 0.25 ml nr. 2,6  $\alpha$ . 3. 0.40 ml nr. 4.

The experiments give an orientation as to the intensity of the effect, but do not allow of a quantitative comparison. Some conclusions as to a possible differentiation between the substances may be drawn:

A methyl group in a 2-position seems to have slightest effect on blood pressure, whereas a methyl group in a 3,4- or 5-position increases the depressant action.

The fall in blood pressure is on an average 50—100 % larger than that caused by procain injected in the same concentration.

of transfer of dyes in connective tissue and this was supposed to be caused by an increased amount of pulsation energy transmitted from the blood vessels. Thus, it follows that decreasing hyperemia must cause a decrease in the rate of dye transfer. Taking the above statement at face value the influence of subnormal and increased blood flow through synovial membranes may in view of the before mentioned dissimilar structural and circulatory patterns induce the following chain of events.

Since the regulatory mechanisms so acting on the vessels as to permit the increased blood flow in hyperemia must be directly opposite in action to those reducing the blood flow to or below the normal level, it seems likely that in the case last mentioned the rate of transfer of soluble matter (colloids, dyes) is decreased and the amount of pulsation energy transmitted to the tissues is diminished. The simplest way in which the blood flow through the synovial membrane can be decreased is by partial constriction of the terminal arterioles. According to the above reasoning this would cause a decreased transfer of matter. The absorption of an isotonic electrolytic solution from the joint cavity to the blood capillaries ought to increase simultaneously owing to a decreased hydrostatic pressure in the vessels engaged in fluid transfer.

On the other hand, hyperemia in synovial membranes — where the capillary bed is so constructed that no shunt mechanisms exist and the terminal arteriole is the most distal arterial vessel — would lead to an increased transfer of colloids through the membrane and a simultaneously decreased fluid absorption to the blood capillaries.

It is known that in resting tissues where a vasomotion mechanism in the sense of ZWEIFACH *et al.* exists, a predominance of the constrictor phase is said to occur in the precapillary sphincters together with a reduced mean flow in the a-v channels. In analogy with the above reasoning additionally increased "vasomotion" would mean that a colloid, e. g. a solute which cannot permeate blood vessels, ought to be absorbed at a slower rate from joint cavities. During increased vasomotion an isotonic electrolytic solution employed as an absorption indicator in the joint ought to disappear at a more rapid rate than in normal controls. In hyperemic synovial membranes the transmitted pulsation energy should — presupposing a vasomotion mechanism as outlined above — increase the transfer of colloid. In the latter case the absorption of an isotonic electrolytic



## b. Combination with Adrenaline.

*Method:* The compounds were tested on their compatibility with adrenaline on the rabbit's blood pressure, mixtures of the compound in a 2 % solution and 2—20  $\mu$ g adrenaline per ml being used, corresponding to a concentration of adrenaline of 1 : 500,000—1 : 50,000 resp. The usual amount of adrenaline added to a local anesthetic for clinical application is 10—40  $\mu$ g per ml (1 : 100,000—1 : 25,000).



Fig. 2. Effect of Compounds nr. 4 and 2,6  $\alpha$  + Adrenaline on Arterial Blood Pressure  
Rabbit 2.0 kg. Time: 30 seconds. 2 % Solutions + 20  $\mu$ g adrenaline/ml.

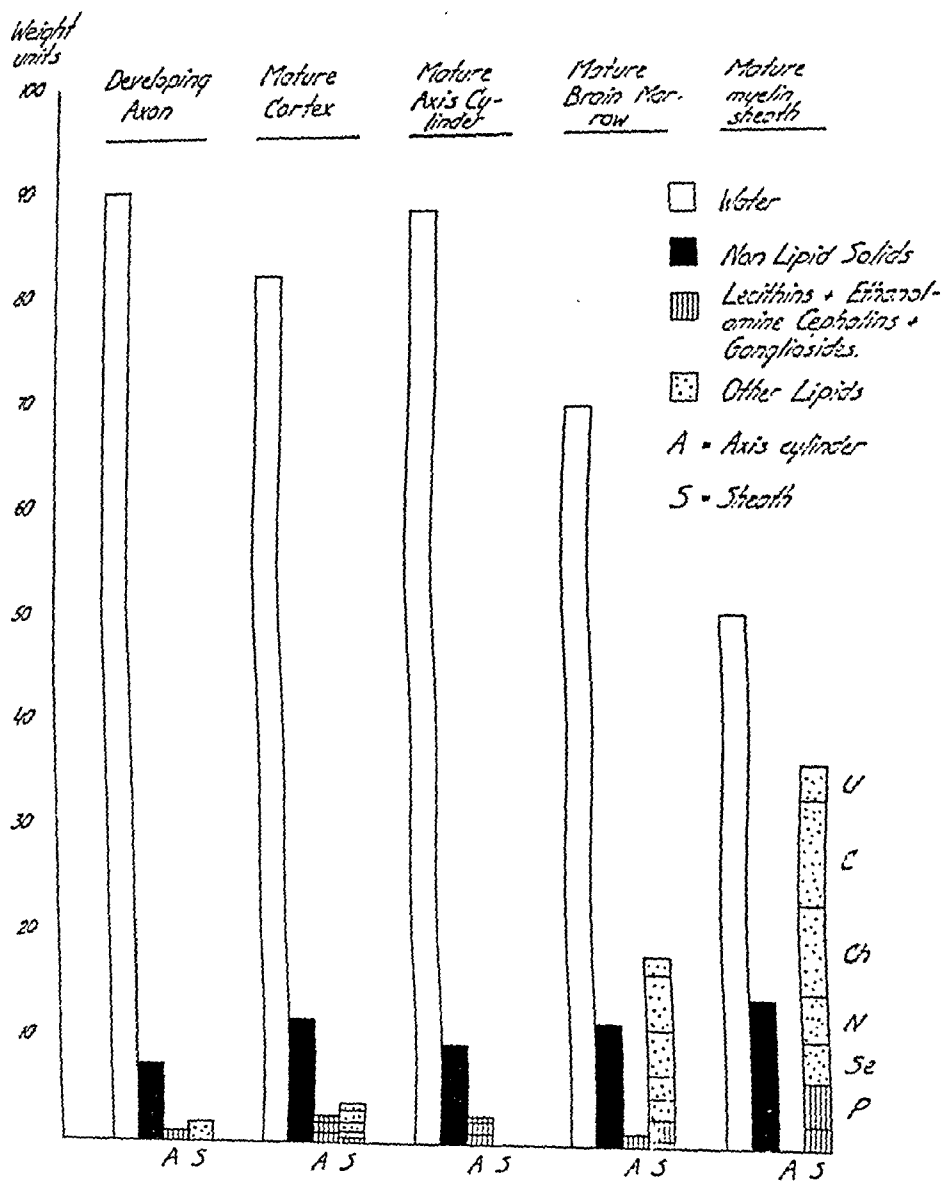
1. 0.10 ml nr 2,6 $\alpha$ .	3. 0.10 ml nr 4.
2. 0.20 ml "	4. 0.20 ml "

*Results:* All compounds gave a rise in blood pressure when using the mixture 1 : 100,000, as an example 0.2 of this mixture, injected into a rabbit of 2 kg, gave a rise in blood pressure of 25—30 mm Hg. Thus, *e. g.* 1  $\mu$ g adrenaline/kg not only counteracted the depressant action of a compound in 2 % solution but even induced a rise in blood pressure. A typical record is shown in figure 2.

Some experiments with 0.2—0.4 ml of a mixture with adrenaline 1 : 500,000 gave a varying response: sometimes a fall and sometimes a rise in blood pressure. This concentration of adrenaline, 1 : 500,000, was thus approximately the threshold value for counteraction.

The experiments suggest the conclusion that those substances, which in pure form caused a large fall in blood pressure, need a greater amount of adrenaline added to give a rise in blood pressure than those substances, which alone gave a slighter fall in blood

Fig. 7



The figures in parentheses represent the approximate contents in % of fresh tissue, calculated on the basis of analyses of the author and others and under the assumption that the nerve cell and the axon contain 88 % water, the myelin sheath 50 %.

The relative importance of various components in axons and myelin sheaths is further illustrated in the diagram, fig. 7, based on similar calculations. There, the lipids — but not the

pressure. A ranking of the substances in combination with adrenaline with regard to these findings gave a fairly good agreement with the ranking of the substances with no adrenaline added.

## Anesthetic Activity.

### *a. Rabbits Cornea.*

*Method:* The possible property of the compounds to induce surface anesthesia was tested on the rabbit's eye. 0.25 ml of a 2 % solution, pH 5.8—5.9, was instilled into the conjunctival sac and left there for 30 seconds. In series I 0.25 ml of a 4 % solution was used too. The onset and disappearance of anesthesia was followed quantitatively in the following way (GOLDBERG, in press):

A series of standardized irritation hairs were used to elicit a blink reflex. The absolute force of the hair was measured. It is shown by the present author (GOLDBERG, in press), that the critical value of stimulation when irritation hairs are used to measure the sensitivity of the cornea to touch or pain stimuli, is the *total* force exerted by the hair *e. g.* expressed in gf, and not the pressure per unit area, *e. g.* expressed in gf/mm<sup>2</sup>. The force of the weakest hair which just elicited the blink reflex was denoted as threshold value: with increasing anesthesia the threshold increases and could thus be used as a measure of the depth of anesthesia.

The course of anesthesia was followed every 15 minutes by applying a whole series of test hairs (30 hairs), ranging from 0.015 to 5.0 gf, the weakest hair being noted, which just elicited the blink reflex. The lid hairs were removed beforehand.

In order to be able to compare different concentrations and compounds, special stress was laid on the following stages:

a) *absolute anesthesia*: no blink reflex could be elicited by any stimulation (threshold stimulus > 5 gf),

b) *relative anesthesia*: threshold stimulus = 1 gf ,

c) *no anesthesia*: the moment when the normal value is reached.

Four eyes on four different rabbits were used for each substance and concentration, as the main purpose was to establish if the substances could bring about surface anesthesia, not to differentiate quantitatively between the substances. Two and two of the substances were applied to the right and the left eye resp. of one and the same rabbit.

There is a large variation from animal to animal, whereas the two eyes of an animal react almost exactly like. This procedure thus allowed a comparison between two substances, and a rough comparison between all of them. In all 116 experiments were performed on 58 animals. The error is approximately 25—50 %.

*Results:* All substances gave a substantial surface anesthesia with total loss of sensitivity to touch or pain stimuli after application of a 2 % solution or a 4 % solution. The absolute anesthesia

the glycolipids, according to FEYRTER, would be more or less diffusely distributed in the axis-cylinder; less is known of cholesterol. However, because of its being a »sheath typical» lipid and its comparatively higher increase than that of the phospholipids in the stage when axons are forming and hence cell surfaces augmenting, at least to some extent cholesterol may be assumed to be concentrated to the surface layer.

Using physical methods, SCHMITT et al. (152) have extensively investigated the organization of the lipids in the *myelin sheath*. Here I have no intention of discussing their well-known results. It should be pointed out, however, that a definite pattern of molecular proportions between the various sheath lipids would logically complete their diagrams of myelin sheath structure. My results suggest that such a pattern really exists, the finer composition of which should be the object of continued studies.

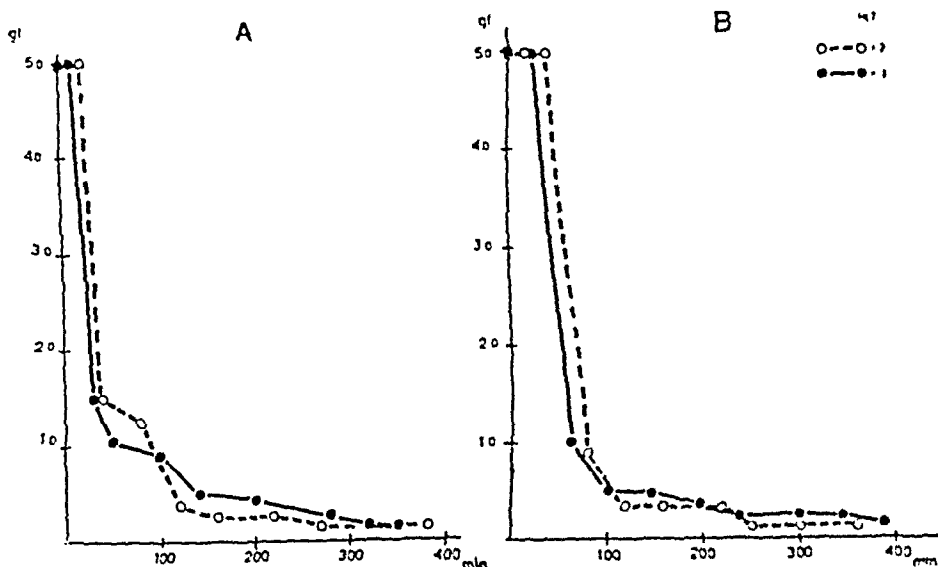


Fig. 3. Surface Anesthesia on the Rabbit's Eye. Compounds nr. 2 and 3. Depth of Anesthesia, established by calibrated irritation hairs, expressed as threshold stimulus in gf plotted against time in minutes.

A. 2% Solutions. B. 4 % Solutions.

lasted at least 12 minutes, in some instances up to 70 minutes, on an average 40 minutes. *Relative* anesthesia lasted up to 5 hours, generally 2—3 hours, and the *sensitivity* returned to normal within  $4\frac{1}{2}$ —7 hours, the anesthesia as a rule lasting longer with a 4 % solution. A typical series of experiments is shown in figure 3.

The record clearly shows the course of anesthesia and the prolongation of the action due to increase in concentration (2 and 4 %, A and B resp.). Compound 2 had a somewhat more potential action than nr 3 in both concentrations, as measured by the longer duration of the absolute anesthesia.

Absolute values are given in table 2. A rough ranking by an arbitrary scale of the substances as to differences in anesthetic property is shown in table 3.

It can be assumed that introduction of two or more methyl groups brings about an increase in the surface anesthetic activity, a methyl group in a 2- or 4-position seems to give a higher activity than a 3- or 5-position; and a 2,6-position to give the strongest effect. A lengthening of the side chain does not change the activity (2,6  $\alpha$  and 2,6  $\beta$ ), whereas introduction of other radicals than ethyl groups at the amino-N (2,6 nb, 2,6 ib, 2,6 ch) definitely decreases the activity.

### Accessories

van Slyke-Neill's manometric apparatus  
 Receiver for 0.5 N NaOH free from  $\text{CO}_2$  (rubber tipped)  
 Quartz lumps crushed to suitable dimensions (1-2 mm in length)  
 Jenna glass reaction vessel with rubber jointed adaptor.  
 Rubber tubing (treated acc. (116))

### Method

*Hydrolysis.* Same as for ethanolamine determination (p. XVII).

*Preparing the sample.* 5 ml sample solution (or sample diluted with distilled water to 5 ml), totally containing 0.03-0.05 mg N as amino acid, are transferred to a reaction vessel and the solution buffered by adding 100 mg citrate buffer.

*Elimination of the preformed  $\text{CO}_2$  gas.* Add some quartz lumps to prevent bumping. Heat to boiling in about 30 seconds the reaction vessel over the flame of a micro Bunsen burner and boil for exactly 1 minute.

Cool solution to below  $25^\circ$  by putting vessel under running tap water for 3 minutes or into ice water for  $1\frac{1}{2}$  minutes.

*Preparing vessel for evacuation following ninhydrin addition.* Fix adaptor rubber tube with squeezer applied to strong water suction pump with interconnected manometer. Moisten free end of rubber tube with distilled water. Now add 2 ml mercury and later 50 mg ninhydrin to cooled solution in reaction vessel. The ninhydrin having been added immediately connect to vessel rubber tube from pump and as quickly as possible and within 30 seconds evacuate to a pressure of 20-30 mm Hg, then close vessel by means of squeezer. Finally, seeing that no air enters, substitute squeezer with glass adaptor. Put away vessels with adaptors downwards, then mercury should cover rubber joint and fill 2 cm of tube above it.

If a series of samples are to be determined it is advantageous to first execute all preliminary steps including evacuation and then boil all the vessels simultaneously.

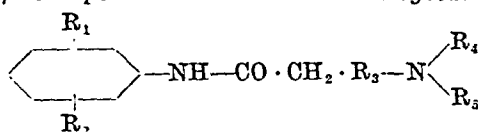
*Boiling samples.* As quickly as possible lower vessels into a large, actively boiling water bath (this should not depreciate temperature below  $98^\circ$ ). Leave vessels completely submerged for 20 minutes.

Cool vessels to room temperature in running water and then put them aside with adaptors down until it is convenient to determine the  $\text{CO}_2$  generated.

*Volumetric determination of  $\text{CO}_2$  generated in reaction vessel* is performed acc. (83). Pressures are measured at a volume of 0.5 ml.

*Blank.* Place 100 mg citrate buffer + 5 ml distilled water in a reaction vessel. Boil for 1 minute, cool, add about 2 ml Hg, evacuate and continue analysis as from «Volumetric determination of  $\text{CO}_2$  generated in reaction vessels».

Table 3.

*Ranking of Compounds as to Pharmacological Properties.*

Substance <sup>1</sup>	Toxicity	Anesthesia		Irritation	
		Cornea	Subcut.	Ear (Rabbit)	Subcut. (Man)
2 .....	+	++	+	+	++
3 .....	+	+	++++	++	++++
4 .....	++	+++	++++	++(+)	++
2,3 .....	++	+++	++++	++	++
2,4 .....	++	+++	+++++	+++	(+)
2,5 .....	+(+)	++(+)	+++++	+++++	++++
2,6 (xylocain) ....	+++	+++++	+++	0	0
3,4 .....	+(+)	++	+++	+++	++
3,5 .....	+	++	+++	+++++	++
2,4,6 .....	+++++	+++	+++	0	(+)
2,6 $\alpha$ .....	+++	+++++	+++++	(+)	+++
2,6 $\beta$ .....	+++(+)	+++++	+(+)	0	++
2,6 nb .....	+++	++	++	+++(+)	+
2,6 ib .....	++(+)	++	++	++	+
2,6 ch .....	++++	++	++	0	+
Procain .....	++(+)	0	+	0	0

<sup>1</sup> Cp. table 1.*b. Intradermal Wheal.*

*Method:* For testing infiltration anesthesia 0.25 ml of a 2 % solution, pH 5.8—5.9, was injected intradermally into the guinea-pig's back. Each compound was tested in 1 %, 2 % and 2 % + adrenaline 1 : 50,000 (20  $\mu$ g/ml). In series I each compound and concentration was tested in 10 wheals, 9 wheals on each animal; in series II 15—26 wheals and in series III 11—16 wheals were made per compound and concentration. All compounds of one and the same series were tested on each guinea-pig, using xylocain and procain as control. The position of each compound and concentration was changed from lateral to medial, from cranial to caudal, thus compounds and concentrations being randomized, all compounds were injected in each animal, procain included, being used as a reference. Experiments have shown that there is a large variation from animal to animal, but a smaller one within one and the same animal between positions. The standard deviation was 12—57 %, and the standard error of the mean 6—8 % on an average. In all 786 wheals were evaluated in 150 animals.

*Anesthetic parameters* (BJÖRN 1947, GOLDBERG 1947 a): The onset of the anesthesia was immediate, therefore only the duration was established by measuring the time till the reaction of the animal to a pin prick became normal. The incidence was 100 %; the range was not tested.

Elements to be added in traces only:

	mg
B	0.05
Cu	0.3
Fe	0.6
Mn	0.06
Mo	0.06
Zn	6.0

These latter elements are added in 3 ml of the following solution:

	mg
Boric acid	57
Ferrichloride. 6 H <sub>2</sub> O	96
Zinc chloride	420
Sodium molybdate	42
Manganese chloride. 7 H <sub>2</sub> O	144
Copper sulphate	375

in 1 l of water

Dissolve all above substances in water and dilute to 3 l volume.

Immediately after preparation decant solution into 3 l liter Pyrex flasks. Stopper flasks with non-absorbent cotton wool, sterilize at atmospheric pressure in autoclave for 15 minutes, then at 15 pounds for 30 minutes or at 20 pounds for 15 minutes. Avoid repeated autoclaving. Discard opalescent, cloudy or coloured solutions.

#### *Preparation of conidium suspension*

Suspend conidia in a few ml sterile distilled water. Take conidia from a culture strain of *Neurospora* about 4-6 days old. Estimate approximate number of conidia per volumetric unit and dilute if necessary. A few 100 conidia per drop is adequate.

#### *Standardizing*

Make up *standard solutions* containing respectively 15, 20, 25, 30 and 35  $\mu$ g inositol per ml saturated NaCl solution.

Pipette into each of several 125 ml flasks 20 ml basal medium and then exactly 1 ml of the respective standard solutions. Charge in this manner 12 flasks or more with each standard solution.

Stopper flasks with cotton wool and sterilize in autoclave. After sterilisation add to each flask 1 drop of sterile conidium suspension. Stopper flasks and incubate at 25° C.

10 and 15 $\mu$ g flasks should be incubated for 6-7-8 days	
20 and 25 " " " " " " " 5-6-7 "	
30 and 35 " " " " " " " 4-5-6 "	

Daily collect in a net mycelia from 4 flasks of each concentration, express liquid between filter papers, roll into separate balls and dry at 100° for 2 hours. Cool in a vacuum exsiccator and weigh to 0.1 mg.



*Results:* The results are given in table 2. All substances were potent local anesthetics, already in a 1 % solution. They were generally more potent in a 2 % solution, and they showed the longest duration when combined with adrenaline. Nr 2 seems to have the shortest duration, and be of the same activity as procain. This result with nr 2 confirms the observations of LÖFGREN (1946 a). He reports that "In Selbstversuchen des Verfassers zeigte sich jedoch nun mehr, dass die Substanz als Infiltrationsanästheticum dem Novocain nahe steht, dass sie aber dem Novocain als Leitungsanästheticum offenbar unterlegen ist." My experiments show that all the other compounds tested cause a definitely larger duration than procain.

As in surface anesthesia, compounds 2,6  $\beta$ , 2,6 nb, 2,6 ib and 2,6 ch have the lowest activity. Then follows a group of 3, 4, 2,3, 2,6, 3,4, 2,4,6, numbers 2,4, 2,5, and 2,6  $\alpha$  having the strongest effect. The differences in effect are not of an order of magnitude, with the exception of the extremes, as to allow of any definite conclusions as to a relationship between chemical constitution and anesthetic activity. Extensive experiments on the relation between 2,6 (xylocain) and procain in dermal wheals definitely show the superiority of xylocain (GOLDBERG 1947 b).

### Irritant Action.

#### *a. Subcutaneous injection into the rabbit's ear.*

*Method:* 0.1 ml of a 2 % solution, pH 5.8—5.9, was injected subcutaneously into the outer third of the rabbit's ear well between the two layers of the skin (GRÖNBERG 1935), albino rabbits being used. This amount produced a slight wheal, which was completely absorbed within a few hours. 5 ears were used for each compound, two compounds on every animal. As for other tests, the animals may vary in their reaction to a compound, an animal sometimes only reacting slightly to a rather irritant compound. It was never noted, however, that an animal was hypersensitive and reacted abnormally to otherwise not irritating compounds. The variations were overcome by injecting two and two compounds in every rabbit. In all 98 experiments were performed in 49 animals. The site of injection was then observed for a total period of 15 days (series I) or 40 days (series II and III) and the following reactions were looked for: hyperemia, extra-vascular blood imbibition, infiltration, surrounding edema and necrosis.

A qualitative gradation is given in table 4, the ranking made at an arbitrary scale according to the number of reactions and their duration.

# EXPIRATORY VOLUMES (STAIR CURVE)

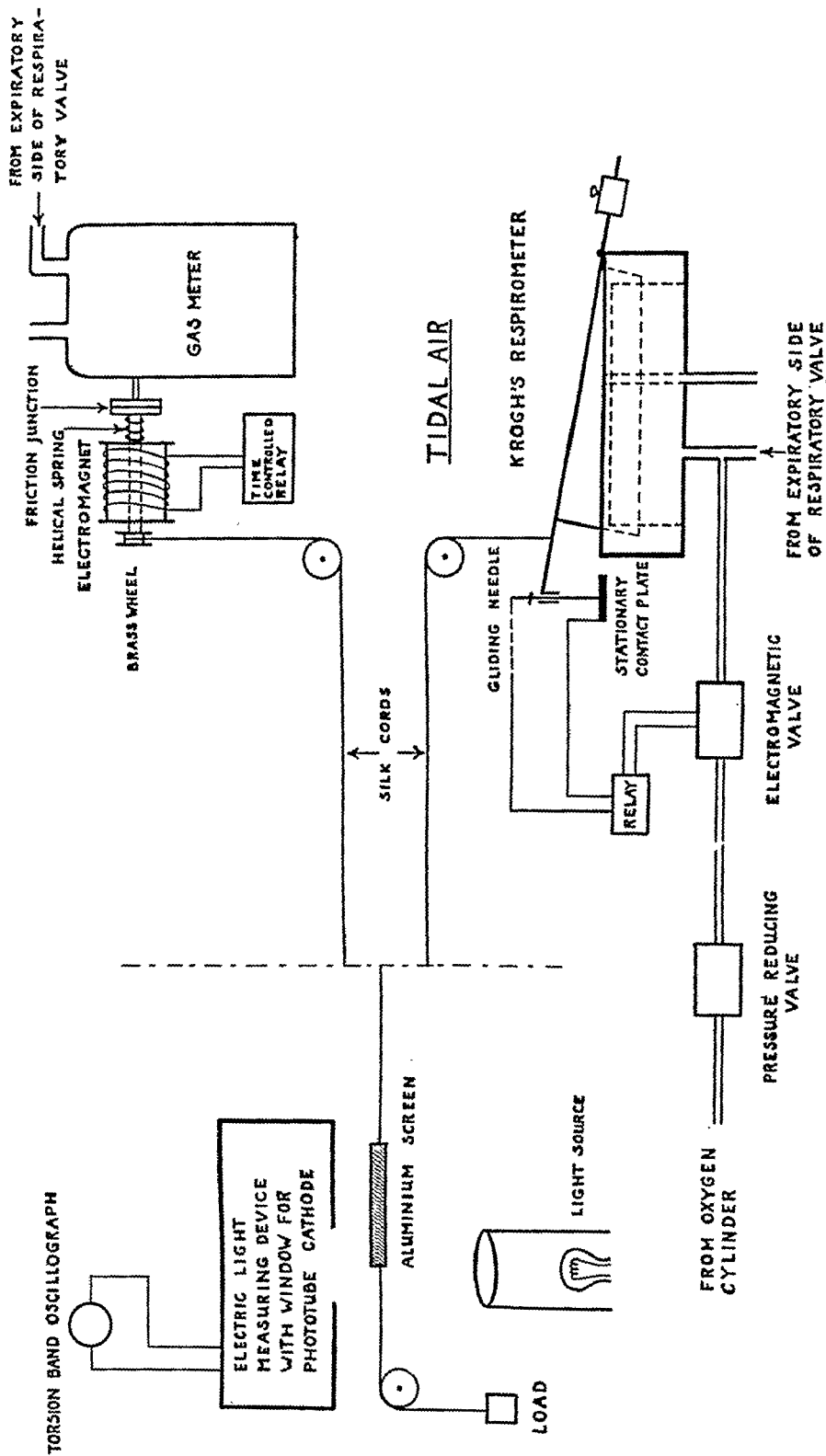


Fig. 2. Methods of measuring and recording the pulmonary ventilation.

Table 4.  
*Irritant Action in the Rabbit's Ear.*

Qualitative Effect					Quantitative Effect
Substance <sup>1</sup>	100 %	50 %	0 %	Ranking	Maximal Area found mm <sup>2</sup> .
2 .....	4	6	7	+	20
3 .....	4-5	8-12	14-15	++	72
4 .....	4-5	8-12	14-15	++(+)	78
2,3 .....	4-5	7-8	12-16	++	45
2,4 .....	7-8	10-12	15	+++	200
2,5 .....	11-14	14-16	21-28	++++	211
2,6 (xylocain) .....	1-2	2-3	4-5	0	6
3,4 .....	6-8	9-20	25-30	+++	78
3,5 .....	20-28	28-35	>38	++++	131
2,4,6 .....	1-2	2-3	4-5	0	6
2,6 $\alpha$ .....	4	4-6	8	(+)	10
2,6 $\beta$ .....	3-4	5-6	7-8	0	4
2,6 nb .....	7-8	10-14	15-25	+++(+)	130
2,6 ib .....	4-5	6-10	12-16	++	45
2,6 ch .....	1-2	2-3	4-5	0	6
Procain .....	1-2	2-3	3	0	6

<sup>1</sup> Cp. table 1.

Hyperemia, possibly infiltration of very slight degree		
	(max.area 2-3 mm <sup>2</sup> )	= 0
"	of slight degree (< 20 mm <sup>2</sup> )	= +
"	+ edema	= ++
" large infiltration + surrounding edema		= +++
" " " " " + necrosis		= ++++

*Results:* A number of experiments on physiological saline, and on procain 2 %, pH = 5.8-6.0, injected in the same way, showed these solutions to give a slight hyperemia and blood imbibition, combined with a slight infiltration of  $1 \times 2$  mm<sup>2</sup>, the reaction lasting in some instances for 2-3 days. These reactions were denoted as 0.

It is obvious that the compounds vary considerably as to possible irritant action: from no effect (2,6, 2,4,6) to necrosis (2,5, 3,5). It seems as if a 2,6-position is a supposition for no irritant effect; other positions cause an irritant action, a single 2-position a slight reaction, a 3- or 4-position an increase. Thus 2,4, 2,5 and 3,4 and 3,5 have the strongest irritant action, 2,5 and 3,5 inducing a necrosis. In 2,4,6 the 2,6-position seems to counteract the effect of the 4-position (cp. injection in man, where 2,4,6 has a slight irritant action). A branching of the side-chain causes a slight effect (2,6  $\alpha$ ), whereas a mere lengthening is without effect

arterial pH during the course of a respiratory cycle (see fig. 5). From these observations it follows, that in conditions with a slow rate of respiration it must be very difficult to obtain single samples of blood, the pH,  $p\text{CO}_2$  or  $\text{CO}_2$  content of which actually represent the average values of the circulating arterial blood. This fact will presumably explain some of the divergences in certain results, which different investigators have obtained from experiments concerning the chemical control of breathing.

### Arterial Oxygen Saturation and pH.

As mentioned the oximeter designed for the recording of arterial oxygen saturation was especially elaborated to compensate automatically for alterations in the hemoglobin content of the blood. It was not possible, however, to attain a complete compensation in case the circulating blood was temporarily diluted excessively by rapidly performed injections of large doses of physiological saline solutions. Then the galvanometer deflection increased temporarily, but returned in a few seconds to its former position.

When acid or alkali was administered rapidly in doses, which induced marked alterations in the arterial pH, the oxygen saturation tracing also showed temporary deviations. The direction and magnitude of these deflections depended upon the previous degree of oxygen saturation. Thus when the animals were breathing pure oxygen, rapid injections of HCl caused a small temporary deviation upwards in the oxygen saturation curve, whereas injections of  $\text{NaHCO}_3$  and  $\text{Na}_2\text{CO}_3$  under the same condition caused a lowering (fig. 7). These colour reactions seem rather obscure in comparison with those observed under hypoxemia. In the latter condition the same injections gave rise to reversed, initial and temporary deviations in the arterial oxygen saturation tracing. These deviations are ascribed to a dominating effect on the arterial oxygen saturation by the well known shift in the dissociation curve of oxihemoglobin, which takes place, when the pH of the blood is changed (cf. BARCROFT & ORBELI 1910—11).

Another example of the mutual relationship between oxygen

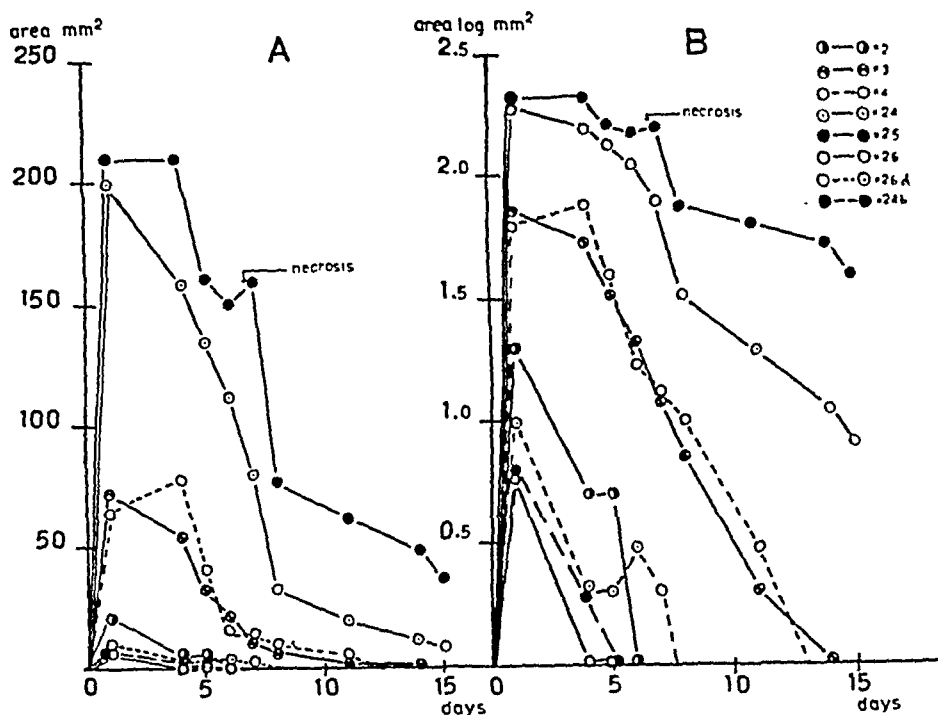


Fig. 4. Irritant Action in the Rabbit's Ear. Area of Reaction plotted against time in days.

A. Area in mm². B. Log. Area.

(2,6  $\beta$ ). A n-butyl radical (2,6 nb) or an isobutyl radical (2,6 ib) at the amino-N cause a strong increase in irritant action, whereas cyclo-hexyl (2,6 ch) has no effect.

A quantitative measure of the degree of irritation was procured in the following way:

The central area of hyperemia + blood imbibition and the area of infiltration were measured and added, the mean of these two areas being used for computation. The average of the 5 reactions was taken as a measure of the irritant effect, and plotted against time. Some examples are given in figure 4.

The record (fig. 4 A) clearly shows that the area, expressed in mm², on any day was related to the irritant effect of the substances, two substances of the same effect showing curves, more or less coinciding (nrs. 3 and 4, 2,6 and 2,4,6), curves for substances of different effect showing a different course (nrs. 2, 2,4, 2,5 and 2,6). For all compounds, with one exception, the maximal area appeared after 24 hours, the horizontal part of the curve of substance nr. 2,5 most probably being due to a missing observation between the 1st and the 4th day. Hyperemia, infiltration and

edema thus showed maximal values after 24 hours; necrosis developed after 4—7 days.

The rate of regression and healing of the reaction is an interesting phenomenon. The course seems to follow a logarithmic or exponential curve, first decreasing rapidly, and then to decrease at a gradually slower rate. A logarithmic course implies the size of the area to diminish progressively by a constant fraction. When plotting the area, transformed into logarithms, against time in days (fig. 4 B), the decrease became approximately linear (fig. 4 B), the regression coefficient, in logarithmic units, on an average being 0,1, corresponding to an approximate diminishing of the area by 20—30 % a day.

The agreement between the qualitative and quantitative ranking is good, and speaks in favour of using these methods as a means of establishing an irritant action. The logarithmic course of the healing of these reactions agrees with theories on the healing of wounds, this too showing an exponential course after a latency period of some days (reviewed by SANDBLOM 1944).

#### *b. Subcutaneous injection in man.*

*Method:* In some tentative experiments each compound was injected in a small amount, 0.1—0.5 ml, intra- and subcutaneously in man in a 2 % solution, pH 5.8—5.9. The subjective reaction was denoted: no pain = 0; slight smarting = +; considerable smarting = ++; intensive smarting, injection to be stopped = +++.

*Results:* The results are seen in table 3. The action varies considerably from no reaction (2,6) to heavy pain, lasting for several days (3, 2,5, 2,6  $\alpha$ ), even after injection of 0,1 ml. Nr. 2,6 and 2,4,6 seem to be the only compounds not giving reaction in man. No relationship of any certainty could be established between the irritant action in the rabbit's ear and that in man. *Thus 2,6 and possibly 2,4,6 are the only compounds passing both tests and giving no irritant action in animal or man.*

### Discussion.

It is evident that there seems to exist a certain relationship between chemical constitution and pharmacological action, this relationship, however, *changing* with the property studied. A survey is given in table 3. No conclusions can be drawn with certainty about the effect of position or radicals not studied,

as the introduction of small changes in the constitution can lead to great effects. Xylocain (2,6) and possibly LL 31 (2,4,6) seem to have outstanding properties, being potent local anesthetics both for surface and infiltration purposes, and not being irritant.

LÖFGREN (1948) has supposed the action of xylocain to be due to inhibited resonance and an o-effect, as judged among other from the molar refraction; the value of the thermo-dynamic ionization constant,  $pK_a$  being low, was supposed to play a certain rôle too. Measurements on isolated motor nerve from frog by EHRENBURG (in press) suggest the free base to decide the activity. Finally determinations of the distribution coefficient oleyl alcohol/water suggest the Meyer-Overton rule not to be valid for this group of local anesthetics (LÖFGREN 1948).

### Summary.

Pharmacological properties of fifteen homologues and isomeres of xylocain, being alkyl-amino-acyl derivatives (table 1) were investigated. All compounds were tested in a 2 % solution, pH adjusted to 5.8—5.9.

The convulsive dose ( $CD_{50}$ ) varied between 0.25 and 0.5 g/kg, the lethal dose ( $LD_{50}$ ) between 0.2 and 2 g/kg; both were established in white mice.

All compounds caused a fall in the rabbit's blood pressure. In combination with adrenaline a concentration of more than 1 : 333,000 (3  $\mu$ g/ml) increased blood pressure.

The compounds were potent local anesthetics, causing a surface anesthesia on the rabbit's eye with an absolute duration of 12—70 minutes and a total duration of 4—7 hours.

The infiltration anesthesia in dermal wheals in guinea-pigs lasted in a 1 % and 2 % solution up to 300 minutes; addition of adrenaline 1 : 50,000 (20  $\mu$ g/ml) caused a marked increase, showing the compatibility with adrenaline.

The irritant effect varied for the different compounds, xylocain and LL 31 (2,4,6) being not irritant neither in the rabbit's ear nor in man.

A relation between chemical constitution and pharmacological action was assumed and varied for each property studied.

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## **“Adaptation Factors” to Weak Light-Adaptation of Isolated Retinal Elements.**

By

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The present work supplements my previous observations on coloured adaptation of single retinal elements of decerebrate cats with a study of adaptation to 'white' light (GERNANDT, 1948). The results can be described very briefly and should be considered in relation to the earlier work. Striking differences had been noted between the effects of a red, a blue and a green adapting light on three test lights, a red at  $0.650 \mu$ , a green at  $0.500 \mu$  and a blue at  $0.450 \mu$  demonstrating again the extreme variability of the isolated elements with respect to colour sensitivity. Sometimes one, sometimes another adapting light caused a large reduction of sensitivity to one or two of the test lights. Sometimes there was a small, sometimes a large general depression of sensitivity to all test lights. The present work is a repetition of the same type of experiment with the same test lights and a 'white' adapting light.

### **Procedure.**

Well isolated large spikes from the dark adapted cat's retina were used and their thresholds first determined for the three spectral test lights, the red, the green and the blue. Then the eye was light adapted for 10 min. with a tungsten lamp run at  $2,800^\circ \text{K}$  the beam of which delivered 800 m. c. at the animal's eye. However, the strength of this light was cut down by means of a neutral filter of density 2.09. Thus

the animal light adapted to about 8 m. c. only. Immediately, after the ten minutes of light adaptation, threshold measurements with the three test lights were begun again and the observation times noted for these thresholds. Gradually the original values were restored while the course of dark adaptation was followed in this manner with the test lights. If an element failed to reach its original value in the dark it was discarded, because in such cases one had to count with some damage caused by the pressure of the micro-electrode.

## Results.

The results will be evaluated in terms of an 'adaptation factor' which simply shows how many times the threshold had risen as a consequence of light adaptation. We shall merely discuss the adaptation factors obtained immediately after light adaptation.

There were 26 elements, 17 of which were on/off-elements for which two adaptation factors were obtained, one for each component (on-component and off-component). Averaging the adaptation factors obtained for each of the three test lights one finds that the green, blue and red test stimuli had been depressed respectively 43, 17 and 10 times. Assuming homogeneous visual purple to be the photosensitive substance concerned, these figures should be equal or slightly higher for red and blue if there were a significant diminution of concentration of this substance. It is clear that neither the red nor the blue part of the spectrum has been activated solely by a photochemical substance with the known properties of visual purple in solution (see GERNANDT, 1948).

However, this rough calculation of average effects does not do justice to the method which, after all, is designed for the analysis of individual elements. It is impossible to show the results for all elements but fig. 1, presenting distribution curves for the adaptation factors of the three test stimuli, should give some idea of the great variation in adaptability. The abscissae show the adaptation factors subdivided into 5 groups plotted logarithmically, the ordinates are number of adaptation factors falling within each of the five groups.

The maximum adaptation factor noted in these experiments was 640 (green-test light), the minimum 0.03. An adaptation factor of 1.0 means that the threshold remained unchanged by light adaptation or returned to normal so quickly that there was no time to record the change. An adaptation factor below 1.0 means

that light adaptation actually made the element more sensitive to the test colour. For two elements there was a *rise in sensitivity* (in addition one similar case had been seen in the previous experiments with coloured adaptation, GERNANDT, 1948). For one of them the adaptation factors were respectively 0.03 (green and blue) and 0.06 (red). The element was dark adapted, recovered fully, and the experiment repeated. Again there was a rise in sensitivity of the same order. It was an on/off-element and the rise referred chiefly to the on-component. For the off-component the values were 1.0 (green), 3.9 (blue) and 0.7 (red), thus no adaptation for green and red and a modest adaptation for blue. The values for the other element were 0.4 (G), 0.7 (B) and 1.5 (R) in the on-component, 41 (G), 10 (B) and 4.4 (R) in the off-component.

The state of balance between excitation and inhibition in the on- and off-components of an on/off-element, expressed by the extremely variable off/on-ratio (GRANIT and TANSLEY, 1948), can apparently be disturbed by a change in the state of adaptation. Some elements which, on our definition (presupposing full dark adaptation and the intensity range available in our spectrum), are pure off-elements are actually on/off-elements with an infinitely high off/on-ratio. This was proved when I found 2 'pure' off-elements in which after light adaptation an on-component turned up. Technically this too is an increase of sensitivity comparable to the one described above. An on-component, suppressed in the dark, has become supra-threshold after modest light-adaptation.

The most remarkable fact, shown by fig. 1, is the extreme variability of the adaptive effect. There are elements which undergo comparatively enormous changes of threshold under the influence of our relatively weak adapting light. As an instance I have chosen two elements for which the adaptation factors were: (i) on-component: 3.7 (G), 15.7 (B) and 18.2 (R), off-component: 640 (G), 300 (B) and 100 (R), (ii) on-component: 340 (G), 25 (B) and 2.8 (R), off-component 376 (G), 39 (B) and 4.3 (R). In the former case the off-component was relatively more adaptable, in the latter case both behaved similarly. In the former case the red in the on-component suffered the greatest amount of adaptation, the blue almost as much, but the green was 'hardy'. With any number of such variations available in my material it is difficult to believe that the photosensitive substance in the receptors always can be the same for the three stimuli. If it be visual purple

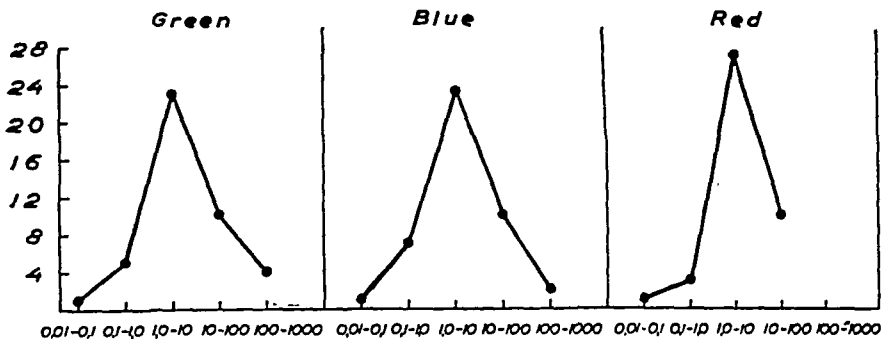


Fig. 1. Ordinates: number of elements falling within each of the ranges for adaptation factors given in log units on the abscissae. The three test colours marked above the graph.

this substance must exist in different states as concluded by DONNER and GRANIT (1948). One of the very interesting off-elements in which an on-component turned up after light adaptation was exceptionally adaptable in the red. The values were: 29 (G), 4.0 (B) and 66 (R). A case of specific blue-sensitivity to adaptation was found in the following on/off-element: on-component: 3.0 (G), 6.3 (B) and 1.2 (R), off-component: 3.7 (G), 106 (B) and 4.3 (R).

The sets of values selected above must not leave the impression that all elements differed as much for the three test stimuli. A statistical analysis would have shown a positive correlation between the degrees of adaptability for the three test stimuli. This, however, was not undertaken. Only the average values given above were calculated.

In view of the sometimes spectacular differential adaptabilities to the three test stimuli in spite of the homogeneity of the adaptation light it is necessary to consider the question as to whether these differences might have been due to differences in the rates of recovery. All three test stimuli could, of course, not be measured simultaneously and thus the relative rates of recovery must have introduced errors. The question is merely whether these errors could have been large enough to explain the results of fig. 1 and the differential adaptabilities noted above.

In the threshold measurements after light adaptation green took the first place in 9 cases, blue in 8 cases and red in 9 cases. The high adaptation factors for red and green were not necessarily among those measurements in which these colours came first. Their distribution was, in point of fact, quite irregular. For blue, however, there was some tendency among the high adaptation factors to occur in the group where blue came first. The times of

recovery varied a great deal, from 10 min. to half an hour or, occasionally, more. The only general rule noted was that the thresholds characterized by large adaptation factors tended to be restored to normal later than those that had changed but little. The more adaptation, the longer the time to full recovery, but this rule was not without exceptions. It is thus quite evident from a close scrutiny of the material that the variations in the adaptation factors, shown in fig. 1, cannot be due to the order in which the measurements have been made.

In my experiments with coloured adaptation it was noted that the adaptability was related to the off/on-ratio of the on/off-elements (GERNANDT, 1948). If the element was relatively more on-sensitive, the maximum effect of adaptation was found in the on-component. Similarly, if it was relatively more off-sensitive, adaptation attacked the off-component more strongly. This rule was found confirmed with the "white" adapting light used in these experiments and for all test lights. It was, in fact, very well obeyed with the green and blue test lights, less strictly so with the red test light.

### Comments.

The variations in adaptability to a white test light of relatively weak strength, shown in fig. 1, may to some extent be due to the variations in the off/on-ratio. This is suggested by the fact that, in general, the effect of the light was felt more strongly by the most sensitive component of the element. A similar explanation is also suggested by the rare cases demonstrating an increase of sensitivity of the element undergoing adaptation. It will be recalled that this increase in two cases led to the appearance of an on-component in what in the dark had been a pure off-element. It is difficult to discard the explanation that in such cases a redistribution of excitation and inhibition within the complex on/off-structure had taken place.

On the other hand, it would certainly be to ascribe too much to the forces maintaining the off/on-ratio if one assumed them to be responsible for the sometimes extremely selective effects upon the three test lights. There must be substances specifically sensitive to red, green or blue light. This was shown very clearly by my previous experiments with coloured adapting lights. We are thus dealing with a complicated system in which specific

coloured effects combine with processes responsible for the distribution of excitation and inhibition within the elements.

Starting, as I have done, with full dark-adaptation one would expect relatively simple results if the current theories, derived from psychophysical experiments, were right in representing this state as one determined by a relatively homogeneous substance, visual purple, such as it is known in the extracted state. The analysis shows a far more complex behaviour of the individual element in modest light-adaptation. My results are thus in full agreement with those of DONNER and GRANIT (1948) who found it necessary to assume that visual purple in the retina existed in states of different probability expressed by different absorption curves. The photochemical experiments of BALL, COLLINS, MORTON and STUBBS (1948) support this explanation. The general and specific variations in the adaptability of the elements are one more expression of a photochemical differentiation which, at the moment, is best ascribed to changes of resonance within the structure 'visual purple-receptor protein'.

### Summary.

Isolated elements in the fully-dark-adapted cat's retina have been subjected to modest light-adaptation of about 8 metres candles for 10 min. and the drop in threshold measured for a red, a green and a blue monochromatic test light.

An 'adaptation factor' was calculated for each test light and element to show how many times the threshold had changed as a consequence of the adaptation.

These adaptation factors varied over a range of about 1—600, in terms of multiples of threshold, both for the different test colours as well as from element to element.

The variation with the test colour demonstrates that the modest light-adaptation used had a differential effect with respect to colour. If visual purple alone were involved this substance must therefore exist in other states of probability than the one determined by the standard absorption measured in extracts from dark-adapted eyes.

The effect of light adaptation on the off/on-ratio shows that the elements also become differently balanced under the influence of light.

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# **Further Investigations on the Transformation of Histidine and Related Substances to Creatine by Animal Tissue in Vitro.**

By

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The present paper is a continuation of two recent notes (STEENSHOLT 1948 a, 1948 b), in which we first investigated the effect of optical isomerism in the transformation of histidine to creatine by animal tissue in vitro, and then attempted to examine some further details of this process. In the present note particular attention will be given to analogous transformations of certain substances which are chemically closely related to histidine.

## **Experimental Results and Their Discussion.**

The biological material mostly consisted of rat muscle tissue, prepared as in the previous experiments.

The histidine, urocanic acid and methionine were all Hoffman-La Roche products. Imidazole lactic acid was prepared from histidine, in the essential points according to the method of FRÄNKEL (1903) and KNOOP and WINDAUS (1904). Imidazole acetic acid was prepared according to the method of PYMAN (for references see BEILSTEIN 1936), which necessitated a rather long and tedious synthetical procedure. The rest of the compound needed for our experiments was kindly supplied by the Research Department of Hoffman-La Roche, to whom I am deeply grateful for their generous help. Both samples agreed with respect to physical characteristics, and no difference between them





and creatinine, and has been used by the present writer in previous work. To begin with, we have therefore investigated whether imidazole lactic acid, imidazole acetic and urocanic acid have any disturbing effect on the determination of creatinine by the B. B. L. E. L.-method. This was done by analyzing pure aqueous creatinine solutions of known concentration and examining the effect of adding the imidazole acids in question. No disturbing influence of the compounds could be detected. We also determined the content of total creatinine in rat muscle pulp before and after addition of the acids. The same total amount of creatinine was always found irrespective of the presence of the acids. Histidine was previously shown to behave similarly. The B. B. L. E. L.-method therefore appears to be sufficiently specific for the purpose in hand.

We now first examined whether urocanic acid can be transformed into creatine by rat muscle tissue in vitro. This was done as follows. A vessel A contained 0.4 g rat muscle pulp suspended in 6 ml phosphate buffer of pH 7.0. Another vessel B was prepared in the same way and contained moreover 30 mg urocanic acid. The mixtures were incubated for 3 hours at 37° C. At the end of this period the mixtures were deproteinated and worked up as previously described, and the determination of total creatinine carried out by the B. B. L. E. L.-method. Urocanic acid was then replaced by imidazole lactic acid resp. by imidazole acetic acid. The results of four experiments of this kind are contained in Table 1, which gives the relative increase, expressed in per cent, of the total content of creatinine in vessel B compared to vessel A. We list at the same time the results of simultaneous experiments with 1(-)-histidine. Experiment 1 was carried out with the same muscle pulp for all four acids, and so were experiments 2, 3 and 4. It may be of interest to know the absolute values of the amounts of creatine in the muscle pulps used in these experiments. We have actually determined the total amount of creatinine, since by our method of analysis all the creatine present is converted into creatinine during the process of autoclaving. In the four experiments listed above we found total amounts of creatinine corresponding to 460, 480, 475 and 468 mg per 100 g of muscle tissue respectively. Remembering the well known fact that creatine and creatinine occur in muscle tissue in the proportion 100 to 1, approximately, it is seen that our values agree well with those given in the literature (see GUGGENHEIM l. c. for figures and references). The variations from one animal to another in our work were therefore rather small. An explanation for this fact may perhaps be sought in the circumstance

Table 1.

	Relative increase in total creatinine in per cent			
	1. exp.	2. exp.	3. exp.	4. exp.
Urocanic acid .....	12.0	14.2	16.0	12.5
Imidazole lactic acid .....	11.0	15.0	15.5	12.0
Imidazole acetic acid .....	3.0	3.2	2.9	2.5
l(-)-histidine .....	12.5	15.5	17.2	14.0

that we have used rats of about the same age and body weight, and, moreover, the animals had been kept on the same diet under identical conditions for several weeks before the experiments.

Table 1 clearly gives an indication of the relative rates of transformation of the imidazole derivatives in question. It appears that there is not much difference between histidine, imidazole lactic acid and urocanic acid, while the rate of transformation of imidazole acetic acid is considerably slower than for the other substances.

We next investigated whether our imidazole acids were actually transformed into creatine, and not into creatinine. The well known fact will be remembered that creatinine, by its structural formula, can be regarded as an imidazole derivative, and it is therefore quite possible to assume a priori that the imidazole acids are converted into creatinine. The B. B. L. E. L.-method is fortunately of sufficient specificity to allow a decision to be reached on this point, since 3,5-dinitrobenzoic acid gives a colour only with creatinine, not with creatine. We need therefore only determine the amount of creatinine in the mixtures before and after autoclaving. Such experiments were already carried out for histidine by MENNE (1942), who found that the content of creatinine in the mixtures before autoclaving was very small and did not change on addition of histidine. The present writer confirmed this and obtained similar results for the other three imidazole acids. Because of the previous work of MENNE the numerical details are omitted. We conclude that all four imidazole acids are converted into creatine, not creatinine.

It is not uninteresting to examine whether methyl donors like methionine, choline and betaine have any effect on the yield of creatine in the process under consideration. This was done by comparing the contents of creatinine in a vessel A containing muscle pulp and phosphate buffer, a vessel B prepared in the

Table 2.

	Relative increase in total creatinine in per cent in vessels							
	B	C	B	C	B	C	B	C
Urocanic acid .....	13.0	12.5	12.0	12.5	14.0	13.7	14.5	14.5
Imidazole lactic acid .....	12.0	12.3	11.9	11.6	12.2	12.1	11.8	11.8
Imidazole acetic acid .....	3.0	3.1	3.2	3.1	2.5	2.5	2.6	2.7
1(-)-histidine .....	14.1	14.1	14.5	14.4	16.0	15.8	16.3	16.3

The total amounts of creatinine in the mixtures correspond to

1. exper.: 475 mg per 100 g tissue
2. " : 500 mg per 100 g tissue
3. " : 490 mg per 100 g tissue
4. " : 465 mg per 100 g tissue

same way but containing also a suitable amount of imidazole acid, and a third vessel C, identical with B but containing the methyl donator. A typical experiment was as follows:

Vessel A: 0.4 g rat muscle pulp; 6 ml phosphate buffer, pH 7.0.

Vessel B: 0.4 g muscle pulp; 35 mg urocanic acid; 6 ml phosphate buffer.

Vessel C: 0.4 g muscle pulp; 35 mg urocanic acid; 35 mg methionine; 6 ml phosphate buffer.

The vessels were again incubated at 37° C for 3 hours and the mixtures then worked up and analysed as previously described. Similar experiments were carried out with imidazole lactic acid and imidazole acetic acid. Table 2 gives the results of 3 experiments of this kind. We include also experiments with 1(-)-histidine, in which case the results agree with those given in a previous note. We give the relative increases in vessels B and C compared to vessel A, together with the absolute values of the total creatinine for vessel A. It appears that methionine has no effect on the yield of creatine. Replacement of methionine by betaine and choline proved ineffective. Experiments at pH 6.0 and 7.8 gave similar results.

These experiments are of some interest for the following reason. If guanidine acetic acid, which has long been recognized as a precursor of creatine, was formed as an intermediary step in the conversion of the imidazole acids to creatine, it is indeed to be expected that the addition of methionine to the reaction mixtures would have increased very considerably the yield of creatine, as is clear from previous work of for instance BORSOOK and DUBNOFF (1940) and the present writer (STEENSHOLT 1945). As was shown

Table 3.

	Relative increase in total creatinine in per cent				
	pH = 6.0	6.5	7.0	7.4	7.8
Urocanic acid .....	3.5	7.0	13.0	9.5	3.3
Imidazole lactic acid .....	3.0	6.8	13.2	9.8	3.0
Imidazole acetic acid .....	0	1.4	3.1	1.2	0
l(-)-histidine .....	2.5	7.2	14.5	7.4	2.0

In the experiment with urocanic acid the total quantity of creatinine in the mixtures amounted to 460 mg per 100 g tissue. In the experiments with the other acids the corresponding figures were 490, 475 and 465 mg per 100 g tissue. The figures in one and the same column are not strictly comparable.

above this expectation did not materialize, and we are therefore probably justified in concluding that guanidine acetic acid is not an intermediary step in the process. It follows that we can hardly regard the conversion of histidine and the other imidazole acids studied here into creatine as a proper methylation process. It may be remembered at this point that the methyl donors examined above are probably the most efficient and certainly the best known ones, as far as present experience goes.

The pH dependence of the transformation imidazole acid-crea-  
tine was studied by making experiments similar to those reported above at pH 6.0; 6.5; 7.0; 7.4 and 7.8. The results of some of these experiments are summarized in Table 3. It appears that there is a pH optimum at about 7.0 for all the acids. This agrees with the previous result for histidine. It is concluded that the differences between the side chains of the imidazole acids studied here are not sufficient to produce any change in the pH optimum.

We may finally mention that we have carried out some experiments on possible inhibitors for the imidazole acid—crea-  
tine conversion. Since imidazole acetic acid was shown above to give only a small yield of creatine, the inhibiting experiments were made only with imidazole lactic acid and urocanic acid as substrates. The procedure was the same as that previously used, for instance in studying the effect of methionine. The results can be briefly summarized as follows. Potassium cyanide, sodium borate and sodium pyrophosphate were used in concentrations up to 0.01 m, 0.01 m and 0.05 m respectively, but could not be seen to affect the process. Sodium fluoride, however, turned out to have an inhibiting effect. When present in 0.01 m concentration

it brought about a reduction amounting to about 50 per cent in the yield of creatine. We also studied the effect of some heavy metal salts, such as manganese chloride and nitrate, ferrous and ferric chloride, and nickel chloride, which were all found to have no influence on the reaction. These results are very similar to those previously found for histidine.

It is tempting to speculate on the possible intermediary steps in the transformation of these imidazole acids to creatine, and it would not be very difficult to write down more or less plausible schemes of reactions. However, it will appear from the above that our present lack of experimental knowledge does not allow us to weigh critically the evidence for or against the various possible hypotheses. It is fairly clear that the process consists in a breaking down (by deaminations and oxidations) of the aliphatic side chain, and in an opening and rearrangement of the imidazole ring. It was shown above that the rate of transformation of imidazole acetic acid is much smaller than that of the other acids, which have a longer side chain. The presence of this side chain may therefore favour the enzymatic cleavage of the imidazole ring, and it is perhaps not unreasonable to suppose that the opening and rearrangement of the ring take place before the side chain is broken down and reduced in length. It was further pointed out that guanidine acetic acid is in all probability not an intermediary step in the reaction. The formation of arginine by the cleavage of the imidazole ring is therefore also excluded, since arginine can be transformed into creatine by muscle tissue *in vitro* via guanidine acetic acid. Hence it seems unlikely that the ring is broken up between atoms 3 and 4, since this might lead to the formation of arginine by the uptake of an amino group. It is possible, however, that the cleavage takes place between atoms 1 and 5, with a subsequent rearrangement during which the methyl group is transferred from position 4 to position 3, and an amino group is taken up. The breakdown of the aliphatic chain then follows. This does not seem an altogether idle speculation, for in some preliminary experiments it was actually found that the presence of ammonium salts increased the yield of creatine. However, these problems require much further work. It is conceivable that the method of isotopes could be fruitfully applied, and experiments with imidazole acids other than those used here might be helpful. The present writer hopes to return to these questions in later papers.

### Summary.

An investigation is made of the transformation of imidazole lactic acid, urocanic acid and imidazole acetic acid to creatine by rat muscle tissue in vitro. The rate of transformation of imidazole acetic acid is much smaller than that of the other imidazole acids and of histidine. The presence of certain methyl donors does not influence the yield of creatine, and it is concluded that guanidine acetic acid does not form an intermediary step in the process. The pH optimum is determined and the effect of certain inhibitors is studied. The possible interpretations of these findings are briefly discussed.

I am glad to express my best thanks to Professor R. EGE for generous hospitality.

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## A Respiration Valve with Insignificant Dead Space.

By

WILHELM VON DÖBELN.

Received 26 November 1948.

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The respiration valves, which are used at present, usually have a dead space, which is considerable compared to the physiological dead space in the respiratory tract. Thus, for instance, the dead space in a commonly used valve is 36 ml. (ENGHOFF 1929). However, in examinations, where refract alveolar air samples are included, it is especially desirable that the dead space of the valve is as insignificant as possible. For practical reasons (respiratory resistance and certain space for the deviations of the valve membranes) the possibilities to diminish the dead space are limited in a valve of the ordinary design.

The constructing of a valve without dead space is possible by dividing the valve so, that inspiration and expiration take place in two tubes independently of each other. This is accomplished by a dividing wall in the valve chamber and the tube for the mouthpiece as fig. 1 shows.

In a valve of the construction described here, each half of the valve is standing in open connection with the respiratory tract during the whole respiratory cycle. Thus a certain change of air may possible take place even in that part of the valve, where the membrane is closed. In other words, a certain functional dead space may exist. Naturally, incomplete and slow closure of the membranes may have the same effect. This also goes for bulging



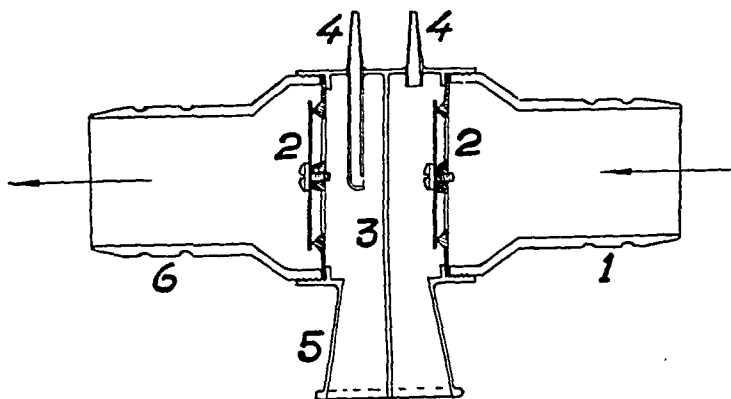


Fig. 1. Respiration valve.

1. Inspiration tube. 2. Rubber membranes. 3. Separating wall. 4. Tubes for air samples etc. 5. Tube for rubber mouthpiece. 6. Expiration tube.

of the membrane and a number of other technical imperfections. However, these defects are the same in the ordinary valves.

With a respiration valve constructed in this manner samples of the air in the inspiration half of the valve chamber were taken refractively at the end of forceful expirations. The  $\text{CO}_2$ -content in the samples was about 1 %. Thus the inspiratory part of the valve in this moment contained alveolar air to about  $1/5$  of the volume. It may be supposed that the inspiratory and expiratory part have functional dead spaces of the same magnitude. Thus the dead space of a valve may be reduced to  $1/5$  of the geometrical volume of the valve chamber and the tube for the mouthpiece by use of a separating wall. In the valve shown on fig. 1 this volume is 50 ml. and the dead space consequently 10 ml.

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# **Interruption of Afferent Pain Paths from the Stomach by Nerve and Root Sections in the Cat.**

By

**K. R. INBERG.**

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Problems connected with abdominal pain are one of the central questions of applied physiology. Opinions still vary as to the rôle of different nervous systems in transmitting painful impulses from the viscera; especially as regards the stomach.

The great interest in vagotomy, shown recently by surgeons has brought into still more prominence the question of interrupting pain pathways from the stomach.

## **Previous Observations.**

As long as we have no definite anatomical knowledge of the visceral afferent fibres, physiological research carried out on animals may prove instructive. So far however the results of such experimnts though similarly planned have often been contradictory.

A manifold sensory innervation of the stomach is presupposed by some researchers but mostly it has been assumed that pain sensations from this organ are transmitted by one nervous system only. For the present it is wiser to keep separate facts derived from human patho-physiology and findings from animal experimentation.

The rôle of somatic nerves is not accurately understood. There are few exceptions. LENNANDER (1901) on the basis of his classical observations at operations on man originated the theory of parietal

referred abdominal pain. He also pointed out the transmitting function of the posterior peritoneum as well. Consequently he thought that the cause of pain in gastric ulcer is lymphangitis spreading in the serous membrane and the retro-peritoneal space.

MORLEY (1931) found not only the anterior parietal peritoneum but also the mesentery decidedly sensitive from its root to a point a little distance away from its attachment to the bowel. These structures are probably supplied by cerebro-spinal nerves. In a diagrammatic figure of his monograph on abdominal pain afferent somatic nerves are shown also in the lesser omentum. Clinical findings correlate with anatomical and experimental facts. SHEEHAN (1933) has studied the distribution of visceroreceptors of the mesentery in man and in the cat. A small number of free nerve-endings appeared to be somatic in origin. It was confirmed by experimental degeneration that the afferent myelinated nerves of the Pacinian bodies travel along the splanchnics.

ISHIKAWA (1932) found the region of the left gastric artery very sensitive in man and the dog. In microscopical examination numerous myelinated fibres of large diameter, very probably belonging to the somatic nerves, were seen, but he thought that these fibres run chiefly in the vagus.

The conveyance of afferent, painful impulses from the digestive tract through the vagi was assumed by many investigators in the 19th century. This opinion later met with opposition but some surgeons continued to believe in it. According to a recent statement of RAY and NEIL (1947) some still think that the vagus nerves do not transmit pain impulses from the abdominal viscera. Clinical observations of the pain abolishing effect of injuries to the spinal cord or spinal anesthesia reaching the upper thoracic segments do not support the opinion about a parasympathetic source of the sensory innervation of the stomach.

Since vagotomy has become popular as an operation for gastric ulcer some authors such as JOHNS and GROSE (1947) point out that the cessation of ulcer pain is the most striking advantage of the operation. I have seen the same effect in two cases as a primary result. Analogously WULFF (1947) reports the complete disappearance of an intractable pain. He admits that this major and dramatic operations may also have some mental influence upon the patient.

Among physiologists also there are still some difference of opinion. According to BEST and TAYLOR (1945) it has not been

definitely demonstrated that the sensation of pain can be transmitted by the afferent fibres of the vagus nerve but it is possible, if not probable, that such is the case. CANNON (1933), however, has stimulated the lower vagus in conscious cats without causing signs of pain.

Some light on the conducting visceral pain has been thrown by balloon distension experiments after destruction of the sympathetic trunks in man for hypertension. BENTLEY and SMITHWICK (1940) observed a disappearance of the pain from homolateral side of the abdomen after unilateral splanchnicectomy and thoraco-lumbar sympathectomy. Pain as a result of balloon distension of the upper jejunum was no longer felt after bilateral denervation. After confirming these observations RAY and NEIL concluded that the passing of pain from the stomach and other abdominal organs in man is solely connected with the sympathetic system. — LERICHE (1937) found that stimulating the splanchnic nerves during an operation under spinal anesthesia was painful.

KAPPIS (1913) stated that in his experiments on dogs the stomach was insensitive to pulling after cutting the splanchnics. FRÖHLICH and MEYER (1912) found in experiments with the same kind of animal no response to stimulation after eliminating the somatic innervation of the abdominal cavity, the splanchnic nerves being left intact.

More recently NASH (1942) says in his "Surgical Physiology" that the afferent path for pain from the stomach is in the splanchnic nerve only. In WRIGHT's (1945) "Applied Physiology" no definite information as to the sensory innervations of the stomach is given.

### Sources of Error.

A comparative study of pain paths from the stomach in primary sensory neurons is possible by resecting alternately the main nerves and posterior roots. By the latter operation — if it has sufficient number of roots — both the afferent fibres from the stomach running in the splanchnics as well as the somatic fibres from the lesser omentum are eliminated. The method has been used before. ISHIKAWA has done a complete series of sections in dogs. His results however are not generally accepted.

Experimental work on pain is not quite successful in animals. As LEWIS (1942) has pointed out the indices of pain in this con-

nection are not very reliable. Some of the bodily reactions recognized by human experience as frequent associations of pain, such as raised blood pressure, withdrawal movements, dilatation of pupil, respiratory changes, and so on are spinal cord or medullary reflexes and can occur without pain. In some phenomena the initiating impulse is supposed not to travel by pain paths. The same may be true in regard to parietal reflexes, which according to clinical experience are far from constant accompaniment of pain and tenderness. A comparative study — not possible in man — might prove informative, presuming it is carried out under conditions as similar as possible. This is to a great extent a matter of stimulation. Three things must be taken into consideration.

Firstly the kind of stimulation is important. The question of an adequate stimulus to organs of the digestive tract which are insensitive to many forms of stimulation is not yet fully settled. Judging from some earlier investigations only the pulling or stretching of the supporting ligaments is painful, but the human and animal experiments of HURST (1911), SCHRAGER' and IVY (1928) and others have demonstrated the pain producing effect of the balloon distension of a hollow viscus. In consequence HURST and STEWART (1929) have emphasized the distension of contraction of the muscular coat of the stomach as an adequate physiological stimulus. This does not mean that other forms of effective stimulation are not possible, as recent observations show the threshold lowering effect of inflammation.

Secondly, the strength of the stimulus must be constant. Neglect of this — together with inadequate recording — is probably the reason for the different results of animal experiments. According to BEST and TAYLOR a pressure reflex can be elicited from every somatic or visceral nerve by stimulation strong enough to cause pain in a conscious animal; a depressor reflex is more likely to be evoked by weak, slowly repeated stimulation. Some variations in blood pressure response have, however, been noted depending on the localisation in the abdominal cavity of the afferent component of the reflex arc. — As it is very difficult to measure the exact strength of pulling I have used the distension method.

Thirdly, a different grade of distension should be used because a difference in the threshold stimulation of sympathetic and somatic nervous systems has been suggested.

## Methods.

The experiments were made on 13 cats. Full-grown animals with a body weight from 2,370 g. to 4,250 g. were used. The experiments include a complete series of operations carried out on four cats belonging to the same litter.

A light ether anesthesia with 10—20 g. ether was induced to make the intravenous injection possible. The recording was done after about an hour — when the smell of the ether had disappeared — in chloralose anesthesia. The amount of drug used was 0.06 g. per kg. in 1 % solution. In rhizotomies 2—3 days previous to the ordinary experiments, a complete ether anesthesia according to the open method with a premedication of 0.125 g. avertin per kg. of body weight was used.

The cat was fixed on its back on an ordinary animal operating table. A heparinised cannula was inserted in the right carotid artery and a glass tracheal cannula in the trachea after ligating the central end. An upper middle line abdominal incision was performed. An inflation tube with a thin-walled rubber bag at the end, fitted airtightly was passed into the stomach and the procedure was controlled through the laparotomy wound. The tube went in the mouth through the hole of a transverse stick which prevented the animal biting it off. The other end of the tube was connected with a mercury blood pressure manometer ("Erkameter"). The carotid cannula was connected through a balanced anti-coagulant fluid system with another mercury manometer and this again with a membrane manometer. Alternatively a U tube with a float was used. The respiratory movements were recorded simultaneously with the blood pressure by means of a KROGH's spirometer suited to experiments with small animals.

I am familiar with cutting of posterior or anterior roots in cats from 26 previous operations for other purposes. I have also a little experience of root and nerve sections in man.

The left and right *vagus* was sectioned according to a technique described recently by several surgeons as suitable in vagotomy, *i. e.*, a trans-hial subdiaphragmatic resection in which the possibility of accessory fibres is observed.

The *splanchnic* nerve was dissected on both sides transperitoneally a little below the point where it curves round the crus of the diaphragm and up to its entrance into the coeliac ganglion. A guide to the nerve was also the suprarenal. Damage to other nerves of the retroperitoneal space was avoided.

In two of the *vagus* and *splanchnic* nerve experiments instead of immediately severing them, a silk thread was led under the nerves and the cutting was done in a later phase with a simultaneous recording of blood pressure and respiration.

The *posterior roots* from 6th (once 5th) to 12th dorsal segments were cut intradurally on both sides in a number of experiments. The sources of the major *splanchnic* nerve in cats are cells in the 5th to 12th dorsal segment. The afferent paths running in this nerve are

believed to enter the spinal cord over fewer segments. In man the segments concerned with the sensory innervation of the stomach are the 7th and 8th dorsal segments and probably the 6th and 9th as shown, for instance, in a table compiled by WHITE and SMITHWICK. The segments in cat (and dog) are however not comparable with those in man as pointed out by FULTON (1943). These ordinary experimental animals possess 13 dorsal, 7 lumbar and 3 sacral segments. — Very little is known about the entrance into the spinal cord of the cerebro-spinal fibres from the lesser omentum. ISHIKAWA states that a section of the 6th to 12th roots bilaterally in dogs causes a considerable degeneration of probably cerebro-spinal fibres around the left gastric artery.

For the purpose of stimulation the rubber bag in the stomach was inflated to a pressure of 30 mm. Hg, 40 mm. Hg and 50 mm. Hg successively. The lowest pressure corresponded to a moderate degree of distension of the organ. The highest pressure meant a very extensive distension of the walls and a considerable stretching of the supporting ligaments of the stomach which was protruding through the laparotomy wound.

### Experimental Results.

Nine experiments successfully carried out are equally divided among sectioning of the vagi, splanchnic nerves and posterior roots. In addition by way of comparison, an equal number of anterior roots were cut in one.

Distension to a certain intra-stomachal pressure was followed by a physiological response, *i. e.*, always by a rise in blood pressure and respiratory changes. Again a pulling of the stomach in a number of my experiments caused sometimes a rise, sometimes a fall of the carotid pressure.

In four cats the stomach was distended in the beginning of the experiments using each grade of the stimulation two or three times. Previously a laparotomy had been performed but the innervation of the stomach was intact in this phase of the experiments. The beginning of the rise in the blood pressure was seen to start immediately after the inflation was induced. The rise went on uniformly reaching its maximum usually within a minute. During the control period before distension the carotid pressure record showed only typical oscillations due to the heart beat and respiration. The fluctuations were about 10 mm. Hg. Higher pressure usually caused a more marked response. — The release of the intra-stomachal pressure may cause some irregularities and always a prompt fall in the carotid pressure. Similar results

Table Showing Rise in Blood Pressure due to Stimulation.

Experi- mental Animal No.	Operation	Rise in Blood Pressure (mm. Hg) <sup>1</sup>		
		Distension of the Stomach to		
		30 mm. Hg Pressure	40 mm. Hg Pressure	50 mm. Hg Pressure
2	<i>Laparotomy</i>	35	45	—
4		25	35	25
7		30	35	40
9		15	20	30
2	<i>Vagus Nerve Section</i>	35	35	40
4		20	25	25
5		45	50	50
7	<i>Splanchnic Nerve Section</i>	10	10	20
8		5	15	15
9		0	10	10
10	<i>Posterior Root Section</i>	0	10	0
11		10	15	20
12		15	15	25
13	<i>Anterior Root Section</i>	15	20	30

were obtained in all experiments of this group. The rise in carotid pressure is tabulated below. The mean values by which the blood pressure exceeds the normal fluctuations are given. They show considerable variations. There are always variations in work on blood pressure and the number of my experiments is quite too small to give any definite report. The general course of the blood pressure response is, however, typical. It is more important to observe the form of the response than the exact increase of carotid pressure.

Respiratory changes due to distension of the stomach were also constant. They are — when fully developed — equal to the changes seen in Fig. 1. Immediately after beginning the distension the respiratory movements which during the control period were regular, became smaller. They were often irregular and sometimes more frequent, followed — when the inflation of the stomach ceased — by one or two large movements which may be compensatory.

After *resection of the vagi* in two cats about the same carotid pressure figures were observed as before. Also in a third animal

<sup>1</sup> The values given in this table are the means of two or three measurements.



B.P. mm. Hg.

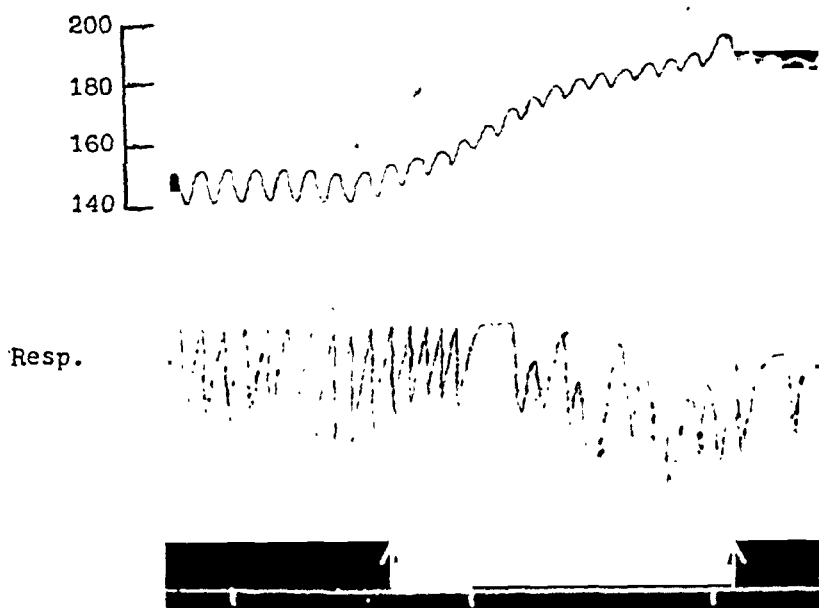


Fig. 1. Experimental animal No. 5. Showing response of carotid pressure and respiration due to distension of the stomach to 30 mm. Hg pressure after vagotomy. The beginning and ceasing of the stimulation at the arrows. Base line, time in 30 secs.

the cutting of this nerve did not inhibit the blood pressure response. The rise was considerable, reaching 45–50 mm. Hg (Fig. 1). In other respect too the response was typical and uniform in all three animals.

In two cats the carotid pressure figures in connection with stomach distension are obviously lower after *cutting the splanchnic nerve* bilaterally than before. In the third animal a smaller rise was also obtained. The less marked blood pressure curve is illustrated in Fig. 2. Otherwise the immediate vascular response was quite typical. The respiratory changes in two cats were unchanged but in experimental animal 8 they were not as clear as usual. In experimental animal 9 an increase of the threshold of the stimulation was noted.

A previous *cutting of the posterior roots* (the vagi being intact) abolished the respiratory response completely in all three animals. A slight rise in blood pressure often remained. The rise is however so slow that it is not seen in the records which consequently appear almost horizontal (Fig. 3). In this experimental group but also after splanchnic nerve section, and after a simple lapar-

B.P. mm. Hg.

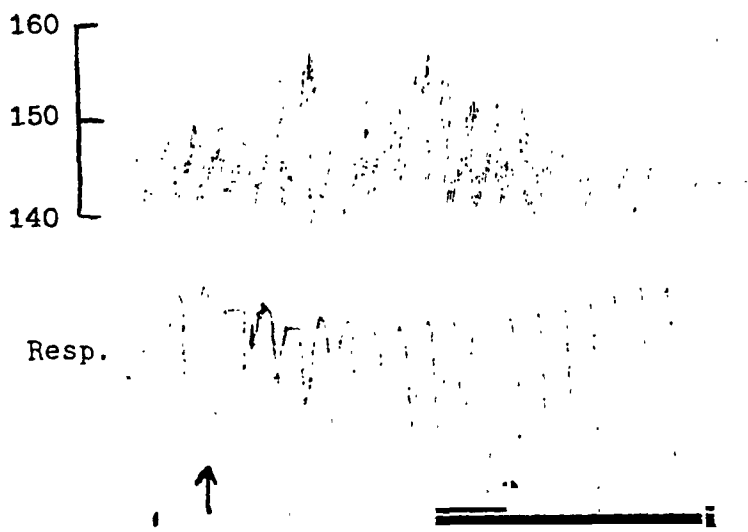


Fig. 2. Experimental animal No. 9. Showing a marked diminution of the carotid pressure rise but otherwise a typical response due to distension of the stomach to 40 mm. Hg pressure after section of the splanchnics. The beginning and ceasing of the stimulation at the arrows. Base line, time in 30 secs.

B.P. mm. Hg.

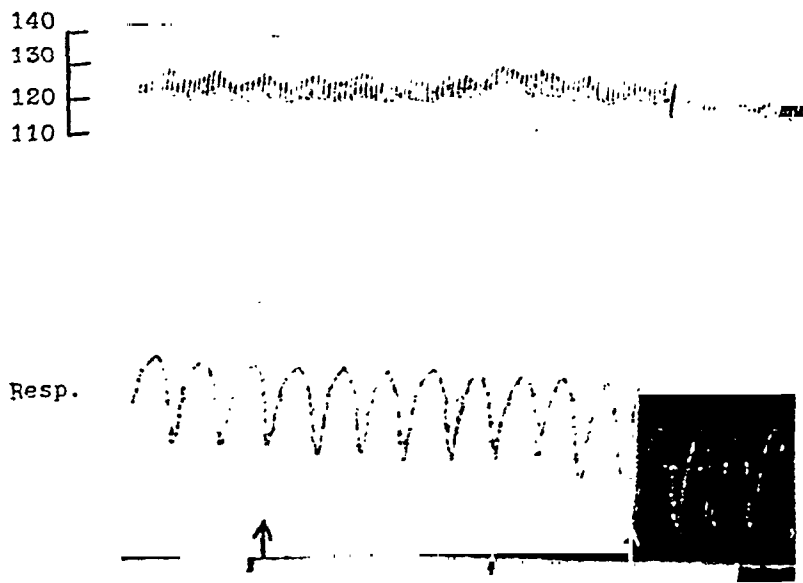
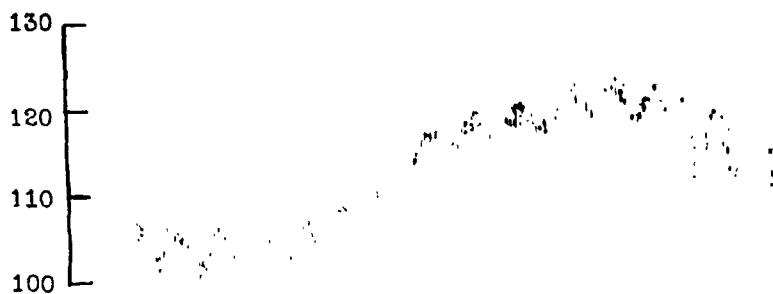


Fig. 3. Experimental animal No. 10. Showing no respiratory response. Blood pressure rise also absent. Distension of the stomach to 30 mm. Hg pressure after posterior root section. The beginning and ceasing of the stimulation at the arrows. Base line, time in 30 secs.

B.P. mm. Hg.



Resp



Fig. 4 Experimental animal No. 13. Cutting of anterior roots. Showing a small but typical vascular and respiratory response to distension of the stomach using 30 mm. Hg pressure. The beginning and ceasing of the stimulation at the arrows. Base line, time in 30 secs.

otomy the oscillations were occasionally small in extent. This seemed not to interfere with the blood pressure rise especially in the beginning of the experiments when there was no obstruction in the fluid system of the recording apparatus. — After a previous section of anterior roots the distension of the stomach gave an immediate rise of carotid pressure. The increase of the blood pressure and the respiratory changes were small but otherwise typical (Fig. 4).

The recording during nerve cuttings gave some interesting results. There was a considerable difference between vascular and respiratory responses to vagus nerve and splanchnic nerve section. Resection of both vagi caused no immediate response. In one cat a slight rise about 5 mm. Hg for a period of  $1\frac{1}{2}$  min. was observed. The other cat showed a considerable but slow rise and fall during 3 min. Occasionally, after the same period had elapsed, an increase in depth and rate of respiration was noted. These changes did not interfere with the acute respiratory response following distension later on during the experiment. The section of both

B.P. mm. Hg.

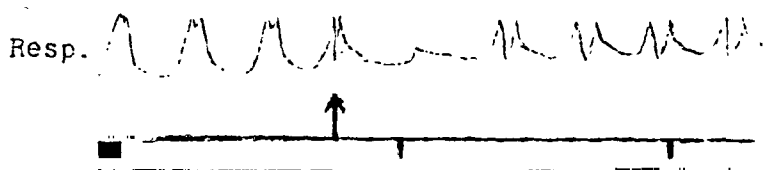
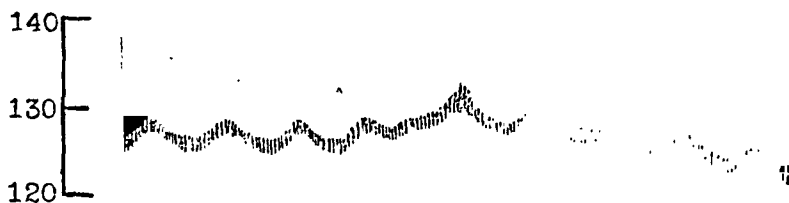


Fig. 5. Experimental animal No. 8. Response due to cutting (at the arrow) of the right splanchnic nerve. Base line, time in 30 secs.

B.P. mm. Hg.

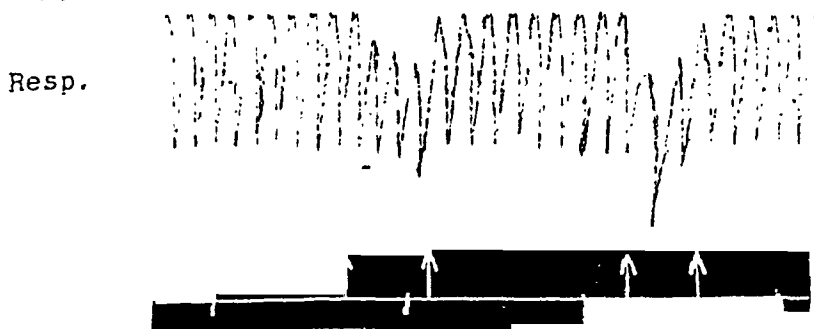


Fig. 6. Experimental animal No. 9. Response due to pulling of the stomach after section of the splanchnics. The interval between the two arrows indicate the duration of the stimulation. Base line, time in 30 secs.

the right and left splanchnic nerve gave a slight but immediate rise (Fig. 5) of the blood pressure similar to the record presented by WRIGHT (p. 531 of his text-book).

Further evidence that blood pressure changes and a typical response do not always correspond was obtained from one experiment in which the rubber bag had accidentally been introduced into the oesophagus — probably in the middle or lower part of it — instead of the stomach. Inflation to a pressure of 30 mm. Hg caused a slow rise of the carotid pressure by about 10 mm. Hg. The respiratory changes were absent. Distension to 40 mm. Hg pressure was followed by a small fall in blood pressure occurring slowly.

In the case of the intact splanchnic nerve (and cerebro-spinal fibres in the lesser omentum) a pulling of the stomach caused a rise in the carotid pressure. After the section of the splanchnic nerve bilaterally the pulling of the stomach caused a fall of the blood pressure and a respiratory response (Fig. 6). After posterior root section no vascular or respiratory response was obtained. In all these experiments the vagi were intact.

### Comment.

The experimental findings of ISHIKAWA differ from my results. He found the stomach otherwise insensitive after cutting the posterior roots of splanchnic nerves, except that pulling the stomach gave some blood pressure response. When these operations were followed by section of the left vagus the response diminished even more but some vascular and respiratory changes remained. He states that severing the vagus causes degeneration of somatic fibres in the anterior gastric plexus.

My results are confirmed by other observers. The experiments of CANNON have been mentioned before. SHEEHAN found no degeneration of the Pacinian corpuscles in the mesentery after severing the vagi but if the splanchnic were cut these nerve endings did not stain. A short period of observation does not yet allow convincing conclusions about a lasting pain abolishing effect of vagotomy in man. It is also possible that the cessation of the important motor function or other sensory functions of the vagi may play a part in the disappearance of pain.

From my experimental results it is to be inferred that probably painful impulses from the stomach in the cat can be conveyed through two different groups of afferent fibres. The intra-dural operation in itself did not abolish the vascular and respiratory response because a similar operation on the anterior roots had no

such effect. A dual transmission of pain from the stomach is only assumed in an anatomical sense. A physiological distinction is not justified. LEWIS has pointed out that the term, sometimes used — “sympathetic afferent” nerves — is incorrect. Afferent pain fibres run only temporarily in the sympathetic nervous system. Anatomically they are comparable to cerebro-spinal fibres.

Observations from operations on man also show that the splanchnic nerve is not the only path for pain from the stomach. Splanchnic anesthesia, according to KAPPIS or BRAUN, in which surgeons earlier used to operate upon the stomach, failed to produce complete insensibility. Pulling the stomach caused a considerable amount of local pain and discomfort. Cerebro-spinal fibres connected with the visceroreceptors in the lesser omentum and probably in the posterior peritoneum and the retro-peritoneal space may not under ordinary circumstances transmit much pain because the end organs are far away. It is however easy to understand that stretching the ligaments or a lymphangitis can cause pain. Lymphangitis is connected with many conditions of the stomach at least in man. LEWIS too admits the possibility of pain from viscera as a result of neighbouring somatic structures being involved. I have previously published some clinical observations on a supposed parietal reference of pain under certain conditions.

To interrupt important afferent paths from an organ certainly diminishes considerably the sensation of pain. LEWIS stresses the fact that pains is to a great extent a matter of spatial summation of pain impulses. According to experimental observations made by him (and KELLGREN) on man and animal, pain or associated phenomena can be derived from any deep-lying tissue. LANGLEY (1900; 1903) realized the relatively small number of the afferent fibres running in the sympathetic system of the abdomen and consequently that the threshold of visceral sensation was high. — The importance of adequate stimulation has already been dealt with before.

A close connection between a viscerosensory “reflex” and a visceromotor reflex has been suggested. An anatomical correspondence of both reflex arcs is not necessary. MILLER and SIMPSON (1924) on the other hand found that in cats the motor responses as a result of the distension of the stomach or stimulation of the gastric sympathetic nerves round the coeliac ganglion, was not entirely abolished by the section of the posterior roots of the 7th to 10th dorsal segments or of the splanchnics.

Extirpation of the coeliac and the superior mesenteric ganglion, however, caused a complete cessation of the reflex effects. SHEEHAN has described, except the two types of receptors mentioned before, in the mesentery a fine plexus of non-myelinated fibres terminating in free nerve endings which appeared to be purely sympathetic.

### Summary.

1. A constant form of stimulation was obtained through distension of the stomach by means of a thin-walled rubber bag inflated to a certain pressure. A different grade of distension was used. Recording of carotid blood pressure and respiration was carried out according to well-known physiological methods.

2. A typical response in its general course followed the distension under light chloralose anesthesia. The absolute blood pressure figures showed considerable variations. A trans-hial subdiaphragmatic resection of both vagi caused no inhibition of the vascular and respiratory response. Cutting the 6th to 12th dorsal roots bilaterally abolished the respiratory response and the immediate blood pressure rise. Some rise of blood pressure occurring slowly often remained.

3. Consequently the splanchnic nerves seems not to be the only path for pain from the stomach. Pain impulses may be transmitted by cerebro-spinal fibres ending in the lesser omentum. A dual transmission of pain from the stomach is only assumed in anatomical sense. A physiological distinction is not justified.

4. Pulling the stomach may cause a rise in carotid pressure by intact splanchnics. A fall in blood pressure could be noted after the same procedure when a number of important paths for pain from the stomach had been interrupted by section of this nerve.

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## **On the Presence of Erythropoietins in the Plasma from Sheep Foetuses During the Latter Half of Gestation.**

By

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### **Introduction.**

The number of oxygen-carrying elements in the foetal blood is known to rise steadily during gestation. In some species as for instance man and ruminants which bear their young in a stage of relatively full development, the foetal blood values near term even exceed those reported during extrauterine life. During the last foetal period signs of increased blood formation are found in the bone marrow as well as in the peripheral blood, which, however, disappear rapidly after birth. (GOODALL, 1908, LIPPMAN, 1924, SEYFARTH, 1927, SABIN et al., 1936.) The foetal erythrocytosis, in infants, according to LIPPMAN and to WEGELIUS (1948) reaches a maximum a few days after delivery. Both authors consider the erythrocytosis during the first hours of extrauterine life to be a real one, and not solely due to haemoconcentration.

Anoxia is generally considered to be the cause of the foetal polyglobulia, as the intrauterine circulatory conditions do not allow a full oxygen saturation of the blood. This view is supported by the negative correlation between the oxygen saturation of the blood in the umbilical artery and the oxygen capacity of the blood, demonstrated by BARCROFT (1946). Differing opinions have, however, been put forward. KRAFKA (1930) observed a decrease in

the percentage of reticulocytes in fetuses shortly before term, and, therefore, regards the foetal anoxia as having no rôle in regulating the erythropoiesis.

In various conditions, where anoxaemia leads to increased blood formation, substances are known to appear, which, transferred into other organisms by means of plasma injections, promote erythrocytosis. These substances, erythropoietins, have been demonstrated in anaemic animals (CARNOT et DEFLANDRE, 1906, GIBELLI, 1911, MÜLLER, 1912, YU-TIN, 1938), in organisms kept under lowered atmospheric pressure (FOERSTER, 1932, YU-TIN, LOESCHKE and SCHWARTZER, 1939, BONSDORFF and JALAVISTO, 1948) and in patients with congestive heart failure (BONSDORFF and JALAVISTO). The plasma taken from babies at delivery or during the first weeks of life, too, is stated to have a similar erythropoietic effect (LOESCHKE and SCHWARTZER, 1939, DÖRING, 1948) while plasma from older, icteric babies is wholly inactive.

On the basis of these results, the formation of such active substances in the foetal organism would be expected.

The present paper is intended to furnish some information as to the presence of erythropoietins in the plasma of fetuses during the later period of intrauterine life, and on the changes in the erythropoietic activity of the plasma during advancing foetal age.

### Material and Methods.

The blood samples to be tested as to the erythropoietic activity, were obtained from sheep fetuses aged from 71 to 121 days. In table 1 are given the age, length, weight and haematological status of the fetuses. The upper and lower age limits are calculated with reference to the time limits, between which conception had taken place; for estimating the probable age the length and weight of the fetuses are considered.

The fetuses were delivered by Cesarean sections. As an anaesthetic for the ewes medinal or nembutal was used. The foetal blood samples, drawn from the carotid artery, or, in some cases, from the umbilical vein, were collected into heparinized centrifuge tubes.

As a control for the foetal blood, samples of adult sheep blood were tested. The blood from pregnant ewes was drawn from the jugular vein under general anaesthesia; as an anticoagulant heparin was used. Blood from non-pregnant sheep, drawn from the jugular vein also, was defibrinated by means of beating.

All the blood samples were centrifuged without delay and 3 cc of the plasma and serum respectively were injected within 3 hours intraperitoneally into adult rabbits.

Table 1.

*Data concerning the sheep foetuses, from which the blood samples were drawn.*

Age of foetuses, days			Length mm	Weight g	Er mill/cu.mm	Hb	L/cu.mm	R %
probable	lower limit	upper limit						
71	71	86	150	150	3.69	49/57	475	9.2
85	85	100	200	300	5.18	56/69	575	8.2
105	99	114	320	1,030	6.35	76/88	775	1.9
106	92	107	330	1,070	5.71	65/75	925	1.3
121	106	121	—	1,960	6.35	76/88	1,450	0.5

The test rabbits were kept in cages, large enough to allow some amount of exercise, and fed on oats, hay, swedes and sprucetwigs. The room temperature was kept low. Blood samples were taken from the ear vein of the rabbits between 9—11 a. m. In the group of rabbits injected with plasma from foetuses or pregnant ewes, haemoglobin, number of red and white cells and reticulocyte percentage were determined before the injection and on the first four or five days succeeding the injection. In those rabbits, which received injections of non-pregnant adult plasma, only haemoglobin and erythrocytes were determined.

The blood picture of the untreated rabbits showed a relative constancy. In single rabbits only slight deviations in the values from day to day were observed. In different rabbits the haemoglobin values varied from 67 to 103 % Hb, the red cells from 4.7 to 5.5 mill/cu.mm, the white cells from 5700 to 7530/cu.mm, and the reticulocytes from 0.8 to 5.4 %.

In order to avoid the confusing effect of protein from foreign species, the rabbits were immunized against sheep plasma. For immunization 0.7 cc. of adult sheep plasma was injected into the ear vein of the rabbit and after 3—5 days another injection of 1.5 cc. was given. After the immunization some inconstancy in the blood values was observed. The plasma-samples to be tested were, therefore, never injected until the recipient rabbit's blood count was found stable again.

A part of the data was treated statistically. For calculating the mean error of the mean, FECHNER's formula was used

$$z = \frac{1.25 \cdot \Sigma \gamma}{(n - 0.2) \sqrt{n}}$$

$\gamma$  being the deviation from the mean of one determination and  $n$  the number of determinations.

### Control Experiments.

In order to exclude the erythrocytosis-promoting effect of adult sheep plasma, the following experiments were performed.

Table

*The variations in the red cell count (Er) and percentage of reticulo-*

Age of foetus, days	Deviation					
	Initial value		1st day		2nd day	
	Er mill./cu. mm	R %	Er mill./cu.mm	R %	Er mill./cu.mm	R %
71	5.5	4.4	+ 0.3	— 1.0	— 0.2	— 2.2
85	5.0	3.5	+ 0.2	+ 0.8	+ 0.5	+ 0.9
85	4.9	2.9	+ 0.1	+ 8.5	+ 0.5	+ 10.5
105	5.4	1.7	+ 0.2	+ 1.3	+ 0.5	+ 6.0
105	4.9	0.8	— 0.3	+ 7.0	+ 0.4	—
105	4.9	0.9	+ 0.3	+ 3.1	+ 0.2	+ 4.1
106	4.9	5.4	+ 0.3	+ 1.2	+ 0.7	+ 1.8
121	5.2	3.9	+ 0.8	+ 3.9	+ 0.4	+ 2.1
$5.10 \pm 0.09$   $2.9 \pm 0.5$   $+0.23 \pm 0.09$   $+3.1 \pm 1.1$   $+0.36 \pm 0.09$   $+4.0 \pm 1.1$						

Column 1: probable age of the fetuses; columns 2—3: initial Er and R values; columns 4—12: deviations in Er and R from the initial value on the 5 first days

1. Serum from an adult, non-pregnant sheep was injected into 3 rabbits.

2. 3 other rabbits received injections of plasma, drawn from 2 pregnant ewes on the 105th and 121st day of pregnancy.

The injection was followed in both groups by a slight rise in the erythrocyte count on the 1st day, which, however, was levelled again on the 2nd day. On the 3rd and 4th day the number of red cells fell below the initial value (Fig. 1, the lower curve). In the haemoglobin only increases or decreases within the limits of normal daily fluctuation were encountered. The number of white cells, when determined, remained unaffected by the injection. The percentage of reticulocytes was slightly increased on the 1st day following the injection, but dropped back to the initial level on the 2nd day (Fig. 2, lower curve).

Accordingly, adult sheep plasma seems, when injected into rabbits, to call forth a slight erythrocytosis and reticulocytosis which, however, last only for one day, and is afterwards replaced by an erythropenia.

### Experiments with Foetal Sheep Plasma.

The erythropoietic activity of foetal blood was tested by injecting plasma from 5 sheep fetuses of different ages into 8

2.

*cytes (R) in the rabbits injected with plasma from sheep foetuses.*

on the

3rd day		4th day		5th day	
Er mill./cu.mm	R %	Er mill./cu.mm	R %	Er mill./cu.mm	
+ 0.1	—	—	—	—	
+ 0.1	+ 5.7	+ 0.0	+ 3.9	+ 0.4	
— 0.2	+ 6.9	—	+ 6.1	—	
+ 0.0	—	+ 0.3	+ 2.3	— 0.2	
+ 0.0	+ 4.2	+ 0.0	+ 6.9	+ 0.1	
— 0.3	—	— 0.3	+ 6.7	— 0.4	
+ 0.1	+ 2.0	— 0.1	—	— 0.2	
+ 0.1	+ 0.7	— 0.5	+ 0.5	—	
—0.02 ± 0.06		+3.5 ± 1.2		—0.06 ± 0.14	

following the injection. In the bottom line are shown the means of the initial values and of the daily deviations, and the mean errors of the means.

rabbits. The data concerning the foetuses are given in table 1. As a rule the injection was followed, in the test rabbits, by an increase in the red cells and reticulocyte percentage, well beyond the daily fluctuation in normal untreated rabbits. The erythrocytosis lasted for 2—3 days, after which the number of red cells was decreased below the initial count. The reticulocytosis, on the contrary, lasted throughout the period of 4 days during which the blood picture was followed. In the haemoglobin only a slight and inconstant increase was found, and, consequently, the color index usually showed a decrease. No increase in the number of white cells were encountered.

The variations in the red cell count (Er) and percentage of reticulocytes (R) in the rabbits injected with plasma from sheep foetuses, are shown in table 2. In the 1st column the probable age of the foetus is given, the 2nd and 3rd column comprise the red cell and reticulocyte values of the recipients on the day of injection (initial value), and the 4th to 12th column the variations from the initial value on the 5 successive days. In the bottom line the means for columns 2—12 and the mean errors are represented. It may be stated that in the number of erythrocytes there is an average increase compared to the initial value on the 1st and 2nd day, which, however, has statistical significance only on the 2nd day. The average increase in the reticulo-

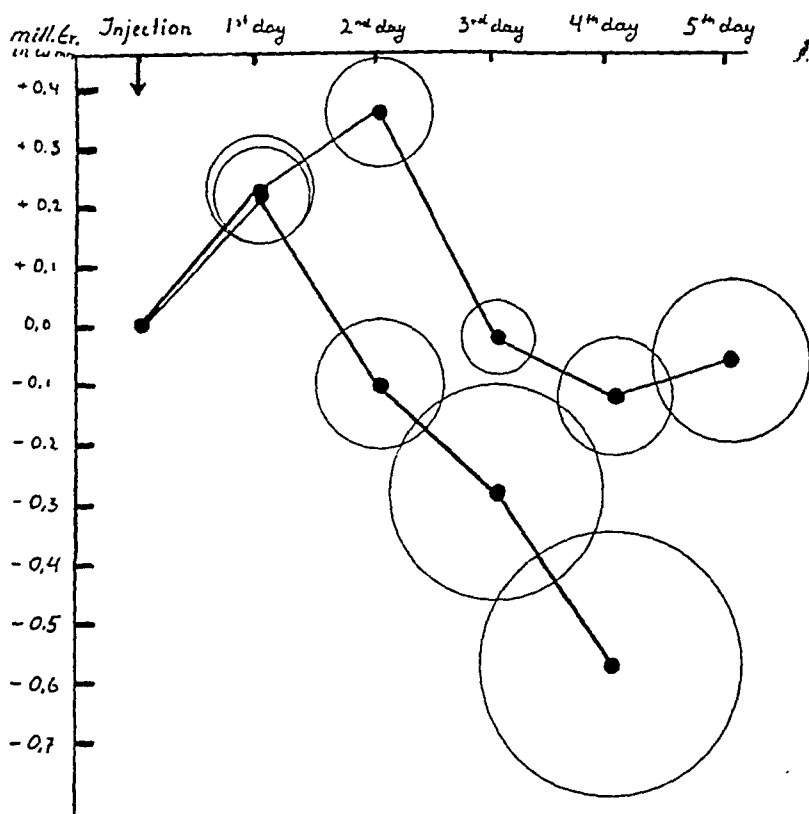


Fig. 1. The effect of foetal and adult sheep plasma on the red cell count of the recipient rabbits. Upper curve: the mean deviation from the initial value on the 5 first days after the injection of foetal sheep plasma; lower curve: the daily mean deviations after the injection of adult sheep plasma. The mean error of each mean is drawn as a circle.

cyte percentage can be held as statistically significant on all 4 days following the injection.

A comparison between the changes in the recipient rabbit's blood picture after injection of plasma from sheep foetuses and from adult sheep is needed for proper evaluation of the polycythaemia-promoting activity of foetal plasma. Fig. 1 represents such a comparison in regard of red cell changes. According to the curves, on the 1st day after injection there is no difference between the effect of adult and foetal plasma, whereas on the 2nd day, when the effect of the foetal plasma is at a maximum, the values in the group treated with adult plasma lie below the initial level. The difference between the two curves at corresponding points on the 2nd day is statistically highly significant. On the other hand, the significance of the difference between both curves on the 3rd and 4th day can only be considered as probable.

### Discussion.

On the basis of the data presented above, it may be concluded, that the plasma from sheep foetuses has a polycythaemia promoting activity during the latter period of intrauterine life stronger than that of the plasma from adult sheep.

The question naturally may be put forward whether the polycythaemic reaction to the foetal plasma is genuine, and caused by an increased activity of the bone marrow of the recipient. Such, probably, is the case. There could hardly be any question of an apparent polycythaemia, due to haemoconcentration, as the recipients, in spite of the erythrocytosis, show no increase in the number of white cells. Furthermore, the decrease in the color index, observed in most cases, indicates the presence of new cells in the peripheral blood. And, finally, the high reticulocyte count lasting several days after the injection must be interpreted as a sign of increased erythropoiesis.

The short interval between the injection and the polyglobulia caused thereby may arouse some doubt as to the origin of the cells. Yet, if the active substances were to cause only an emptying of the blood depots, the reaction could not very well last as long as 2 days. On the other hand, signs of increased bone marrow activity are usually not considered to appear in the peripheral blood as early as the 1st or 2nd day. According to WHITBY and BRITTON (1947), the reticulocyte peak in pernicious anaemia patients occurs on the 3rd to 10th day after the onset of liver treatment, and JACOBSON and WILLIAMS (1945) working on splenectomized rabbits, report an increased percentage of reticulocytes on the 2nd to 6th day after intravenous administration of active liver substances. The early erythrocytosis and reticulocytosis in the present material is, however, in good accord with the results of some other authors. HAHN, ROSS, BALE and WHIPPLE (1940) detected marked iron-atoms in the circulating red cells of anaemic dogs a few hours after the feeding, and KARVONEN (1948) in his calculations arrives at maturation times for erythrocytes of a few hours.

The rapid decrease in the red cell count after the initial erythrocytosis might possibly seem unexpected. Still, the reticulocyte percentage remains high for several days, and even the decrease in the erythrocytes is smaller in the group of recipients treated

with foetal plasma than in the control group. Any lasting effect, on the other hand, could hardly be expected after a single injection of a substance which probably is soon destroyed or inactivated by the recipient organism. — The recipients, it must be born in mind, are normal animals, with a balanced blood status, and naturally would strive to level their blood count as soon as the stimulus had disappeared.

The fall in the number of red cells below the initial value, observed in the main experimenteal group as well as in the control group, remains unexplained. It is not very likely that the frequent removals of blood would be responsible for the anaemia, the amounts drawn being very small. Another explanation might be the toxic, anaemia-producing effect of foreign proteins. This is all the more likely as there was no control to show whether full immunity was obtained by the 2 injections of sheep plasma previous to the experiment. The effect of heparin, used as an anti-coagulant, upon the blood picture, is an unknown factor, too.

As the rise in the erythrocyte count and percentage of reticulocytes even in the control group on the 1st day after injection is within the limits of statistical probability, it must be regarded as likely that normal sheep plasma has a slight polycythaemic effect on the rabbit. Some small deviations in the red cell count and percentage of reticulocytes after the injection of adult sheep plasma, might, however, be regarded as non-specific reactions to foreign proteins, due to lack of immunity.

As to the permeability of the placenta to erythropoietins, nothing definite can be said. In view of the difference between the erythropoietic activity of foetal and maternal plasma, there probably is no complete permeability. The slight polycythaemic reaction caused by the injection of pregnant ewe plasma, cannot be regarded as due to foetal erythropoietins, as plasma from non-pregnant sheep gives a similar reaction. On the other hand, considerable amounts of foetal erythropoietins might be present in the mother's circulation, without their concentration yet being high enough for 3 cc. of the plasma to cause a noticeable polycythaemic reaction. Furthermore, the higher oxygen-content of the maternal blood would possibly lead to a rapid inactivation of the foetal erythropoietins. On the other hand, the erythropoietins might be inactivated by a special activity of the placenta, or they might fail to pass through placenta because of a large molecular size.



The red cell and haemoglobin values for the foetuses in the present material, in accordance with the results of previous investigations show a rise towards term (Table 1). This rise does not disagree with the view, suggested by fig. 3, that the erythropoietic activity of the plasma increases with growing foetal age.

The fall in the percentage of reticulocytes during the last period of gestation, at the time when the number of red cells increases and the plasma contains erythropoietic substances (table 1, last column) might seem difficult to explain. Yet, it must be remembered, that in sheep blood during extrauterine life only an occasional reticulated red cell is encountered. Consequently, the decrease in the reticulocyte count need not be considered as a sign of decreased blood formation, but can be regarded as an initial development towards adult state.

Considering the data known about the foetal blood formation and the peripheral blood picture of foetuses, it seems most probable that the erythropoietic substance present in the plasma is at least partly responsible for the foetal polyglobulia. The existence of a humoral stimulus for the foetus's bone marrow is also presumed by HOOGSTATEN and KARVONEN (1948) who could not detect any difference between the erythropoietic activity of the bone marrow from the cranial and caudal end of the foetus, regardless of the higher oxygen tension in the cranial end.

### Summary.

In order to demonstrate the presence of erythropoietins in foetal plasma, the following experiments were performed.

1. Controls. Plasma from pregnant ewes and adult, non-pregnant sheep was injected intraperitoneally into 6 rabbits, immunized against sheep plasma. The adult plasma caused a slight erythrocytosis and reticulocytosis on the 1st day after injection, which was on the 2nd day replaced by erythropenia, with a normal percentage of reticulocytes.

2. Plasma from 5 sheep foetuses aged from 71 to 121 days was injected into 8 immunized rabbits. An erythrocytosis was observed on the 2 first days after injection, and a remarkable increase in the percentage of reticulocytes took place. The red cell values were decreased below the initial level on the 4th and 5th day, though never as low as in the control group on corresponding days. The reticulocytosis lasted for 4 days. The increase

in the red cells and reticulocytes compared to that in the control group was found statistically significant.

It is concluded, that the foetal plasma near term is more active in promoting erythrocytosis than adult plasma. The active substance contained in the plasma probably has a rôle in stimulating the foetuses' bone marrow.

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## Effect of Dihydroergotamine on the Oxygen Consumption.

### Experiments on Cats.

By

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In a previous work (LUNDHOLM and MOHME, 1949) it has been shown that ergotamine in a dosage of 25  $\gamma$  per kg of the body-weight reduces the oxygen consumption on an average by 13 per cent. In order to ascertain whether this reduction of the oxygen consumption may be attributed to the sympathicolytic effect of the ergotamine, the corresponding effect on the oxygen consumption of dihydroergotamine (DHE), prepared by STOLL and HOFMANN (1943), has been investigated. In fact, according to ROTHLIN and BRÜGGER (1945), BRÜGGER (1945) and ROTHLIN (1946/47), DHE has a more powerful sympathicolytic effect than ergotamine whereas its direct effect on the organs is weaker.

*Method.* The oxygen consumption has been recorded in accordance with a technique previously described (LUNDHOLM and MOHME, *loc. cit.*). The investigation was made on six cats; it comprised 2 experiments with DHE and 1(—2) control tests with the injection of physiological saline into each cat. The procedure was as follows: When the cat had been introduced into the respiration box and had come to rest (after about 20 minutes), the oxygen consumption in the course of 60 minutes was recorded (normal test). An intramuscular injection of DHE, in physiological saline, was then given in a dosage of 25  $\gamma$  per kg of the body-weight. This dosage was selected, firstly because previous tests with ergotamine had been made with the same amount, secondly because any specific effect of DHE should be shown even

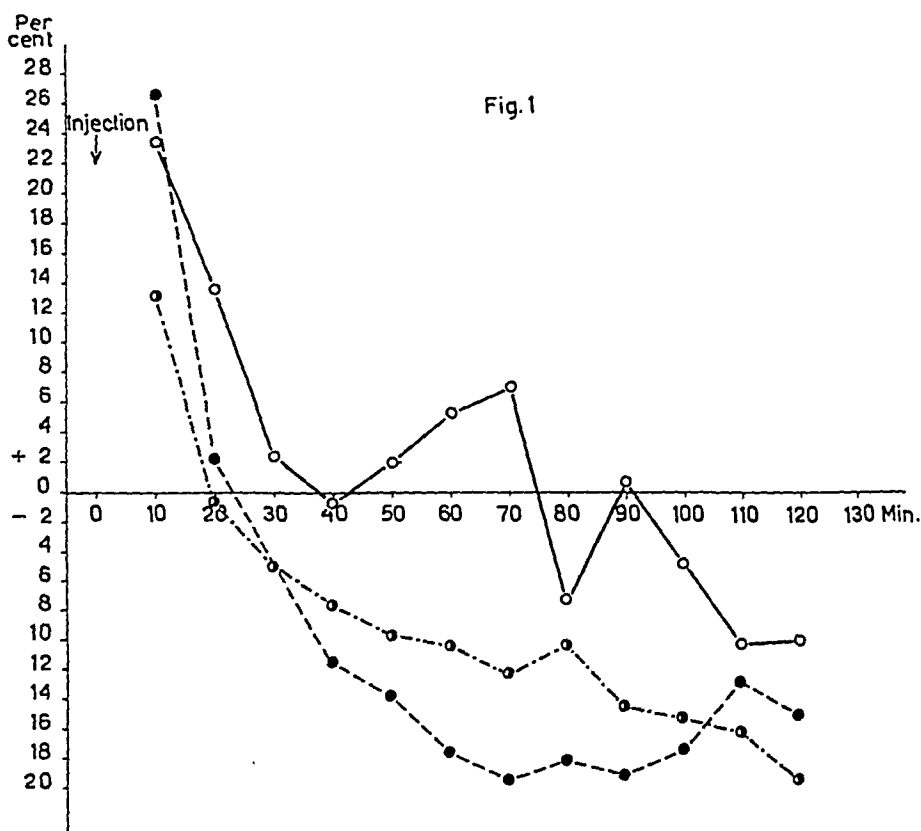


Fig. I. Time-action-curve of DHE and ergotamine and control tests.

Abscissa: Time in minutes after injection.

Ordinata: Increase or decrease per cent of the oxygen consumption.

○ — — — ○ — — — ○ DHE (25  $\gamma$  per kg, intramuscular injection).

● — — — ● — — — ● Ergotamine (25  $\gamma$  per kg, intramuscular injection).

○ — — — ○ — — — ○ Control (physiological saline).

with a comparatively small amount of the substance, and lastly because it was desirable to avoid toxic effects. After the injection the consumption of oxygen from 5 to 125 minutes was again recorded. — In the control tests the procedure was the same, except that physiological saline was injected instead of DHE.

*Results.* The time within which DHE and ergotamine, respectively, have their effects on the oxygen consumption is recorded by the chart in Fig. I. The ergotamine values have been taken from an earlier work (LUNDHOLM and MOHME, *loc. cit.*). The chart shows that the effect of the drugs on the oxygen consumption sets in after the lapse of about 30 minutes. This tallies with ROTHLIN's statement (1947) that DHE is not completely absorbed until after ca. 30 minutes. Not, however, until about 50 minutes

Table I.

Cat number	Cat weight g	Dihydroergotamine			Control		
		normal O <sub>2</sub> cc/60 min.	drug O <sub>2</sub> cc/50—110 min.	increase or decrease per cent	normal O <sub>2</sub> cc/60 min.	phys. sal. O <sub>2</sub> cc/50—110 min.	increase or decrease per cent
I	2 350	1. 861.5 2. 922.3	832.7 821.8	— 3.3 — 10.9	861.1	956.1	+ 11.0
II	1 950	1. 1 031.5 2. 1 016.4	913.7 948.9	— 11.4 — 6.6	934.3	924.2	— 1.1
III	1 350	1. 624.3 2. 745.2	599.4 615.9	— 4.0 — 17.4	725.0	670.1	— 7.6
IV	3 300	1. 1 199.2 2. 1 468.9	1 104.4 1 302.1	— 7.9 — 11.4	1 312.0	1 340.9	+ 2.2
V	2 100	1. 1 108.7 2. 938.9	1 063.7 809.3	— 4.1 — 13.8	915.6	850.4	— 7.1
VI	2 800	1. 1 263.2 2. 1 480.5	1 219.3 1 273.1	— 3.5 — 14.0	1 870.8 1 190.2	1 996.0 1 188.9	+ 6.7 — 0.1

The effect of 25  $\gamma$  per kg body weight of DHE of the oxygen consumption and control tests. The oxygen consumption is recorded 50—110 minutes after injection.

have elapsed since the injection do we notice a distinct fall of the oxygen consumption, which reaches its minimum (— 19.4 %) during the second hour. In Table I, where the results are given in figures, the oxygen consumption during the time 50—110 minutes after the injection is accordingly recorded. The change as compared with the normal values has been estimated in percentage.

A study of the Table shows that in all the tests a fall of the oxygen consumption has set in. This fall averaged 9.0 per cent. Expressed in cc per kg of the body-weight the fall will be 43. In the percentage computation the range of variation was — 3.3 % to — 17.4 %. (Computed in cc per kg of the body-weight, the corresponding range of variation is — 12.3 to — 95.8.)

The material has been treated statistically in accordance with the *t*-test (FISCHER 1936). The results will be found in Table II. The fall of the oxygen consumption after the administration of DHE is statistically significant. In the control tests no such effect can be observed. The difference between the control tests

Table II.

Variate	Mean Increase or decrease per cent	Standard error of the mean	Degrees of freedom	t	P
Dihydroergotamine .....	— 9.0	± 1.38	11	6.529	< 0.001
Control .....	+ 0.6	± 2.57	6	0.233	> 0.2
Ergotamine .....	— 13.4	± 1.83	9	7.341	< 0.001

Difference		Standard error of the difference	Degrees of freedom	t	P
between control tests and dihydroergotamine tests ..	+ 9.6	± 2.920	17	3.287	0.001—0.01
between dihydroergotamine and ergotamine tests .....	+ 4.4	± 2.294	20	1.918	0.05—0.10

Statistical analysis according to the t-test of dihydroergotamine, ergotamine and control tests. (The ergotamine tests have been hold from LUNDHOLM and MOHME, 1949.)

and the dihydroergotamine tests is also very probable. No significant difference in the effects of DHE and ergotamine on the oxygen consumption can be shown. To judge by Fig. I, the effect of ergotamine seems to be somewhat stronger. No reliable conclusions can, however, be drawn, seeing that towards the end of the recorded tests the DHE curve also fell to the same low values as the ergotamine curve.

*Discussion.* DHE, as previously mentioned, has the sympatholytic effect of ergotamine in an intensified degree, whereas its direct effect on the organs is weaker. As DHE too tends to lower the oxygen consumption and as this fall is of the same magnitude as after the administration of ergotamine, it may presumably be attributed to an inhibition of the impulses from the sympathetic system. This view is borne out by the effects of other sympatholytic drugs on the oxygen consumption. According to MUTAKAMI (1930), yohimbine lowers the oxygen consumption in rabbits. ISSEKUTZ and HARANGOZÓ-OROSZY (1942)

found that F 883 in a dosage of 0.1 mg per kg lowered the oxygen consumption in rats by ca. 15 per cent. According to BJORCK and HALL (1947), dibenzyl- $\beta$ -chloroethylamine (30 mg per kg) lowers the oxygen consumption in rats by 17 per cent. The effect of all the sympathicolytic drugs on the oxygen consumption is thus both qualitatively and quantitatively the same. According to CANNON, NEWTON, BRIGHT, MENKIN and MOORE (1929), a fall of the oxygen consumption by 10 per cent. ensues in cats after complete sympathectomy. The low values found for the oxygen consumption after the administration of DHE and ergotamine, — 9.0 and — 13.4 per cent., respectively, are also well compatible with the supposition that the fall of the oxygen consumption is caused by blocking the impulses from the sympathetic system.

### Summary.

The effect of dihydroergotamine (DHE) on the oxygen consumption has been investigated by the author in experiments on cats. When about 30 minutes have elapsed after the injection of DHE in a dosage of 25  $\gamma$  per kg of the body-weight, the effect sets in. About 50—110 minutes after the injection the oxygen consumption falls on an average by 9.0 per cent. The fall of the oxygen consumption is attributed to an inhibition of the normal activity of the sympathetic system.

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# **Impulses from a Cutaneous Receptor with Slow Adaptation and Low Mechanical Threshold.**

By

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Received 4 January 1949.

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## **Introduction and Problem.**

In an electro-physiological investigation on *Nervus cutaneus surae fibularis* (below called the sural nerve) in the rabbit it was found that mechanical stimulation of the skin could give rise to nerve discharges which were slowly adapting. The present work describes the function and distribution of the end organs responsible.

## **Historical Section.**

In 1926 ADRIAN and ZOTTERMAN showed on cats that impulses can be recorded from a cutaneous nerve when a mechanical stimulus is applied to the hairs or to the foot pad in the distribution area of this nerve. Two different kinds of response are described, a short outburst of impulses when the hairs are rapidly bent and kept in this position and a continuous discharge when a heavier pressure is applied to the foot pad. The former type of discharge was elicited in hair touch receptors which thus have a rapid adaptation to a constant mechanical stimulus. Pacinian corpuscles or similar end organs were held responsible for the continuous discharge recorded when a heavier pressure was applied to the foot pad. These end organs were thus evidently slowly adapting. These findings have later been verified, those concerning the hair touch receptors by ZOTTERMAN (1933, 1937, 1939 a, 1939 b), those concerning Pacinian corpuscles by ADRIAN and UMRATH (1929).

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FITZGERALD (1940) recorded impulses elicited in slowly adapting receptors around the vibrissae hairs of the cat.

The neuro-histology of the sural area in the rabbit has been especially studied by WEDDELL, GUTTMANN and GUTTMANN (1941). Two types of receptors were found in this area, basket-formed end organs around the root of the hairs and beaded fibres. The former are innervated by myelinated, the latter by unmyelinated fibres so far as can be judged from the histological picture. No Pacinian corpuscles were ever found in this area. This is, as will be seen, an essential point and has during the present investigation been histologically controlled (FRANKEN-HAEUSER, WEDDELL and FEINDEL 1949). Nor has there hitherto been found any other organized end organ in the region.

### Technique.

*Preparation.* Young rabbits (800—1,800 gr.) under urethane narcosis were used for the experiments. The sural nerve was exposed either in the middle of the thigh or below the knee or, occasionally, in both these places and prepared free from surrounding fat and loose connective tissue. The epineurium was usually left intact.

The blood supply to the nerve was kept intact as far as possible. A longitudinal vessel had to be destroyed when the nerve was exposed in the thigh and a few small vessels when the incision was below the knee.

The nerve was prevented from drying by keeping it either in moist air or in medicinal liquid paraffin. Tyrode solution was also used for this purpose.

*Recording device.* The impulses were led off with silver wire electrodes from the exposed nerve. Two four-stage condenser resistance push-pull-coupled amplifiers were used. The impulses were made audible by a loudspeaker and visible on cathode ray tubes. Records were taken on continuously running bromide paper.

*Stimulation.* The cutaneous receptors were stimulated either by touching the hairs with cotton wool or by touching the skin with a blunt rod or a no. 1 nylon suture thread on a holder. A thrust of approximately 200 mgr. bent the nylon thread. The diameter of the thread was 0.15 mm. In some experiments the hairs were cut short in order to expose the skin in the sural area.

### Results.

It is well known that the receptors at the base of the hair adapt rapidly to a constant stimulus (see historical section). This was repeatedly verified in the present investigation.

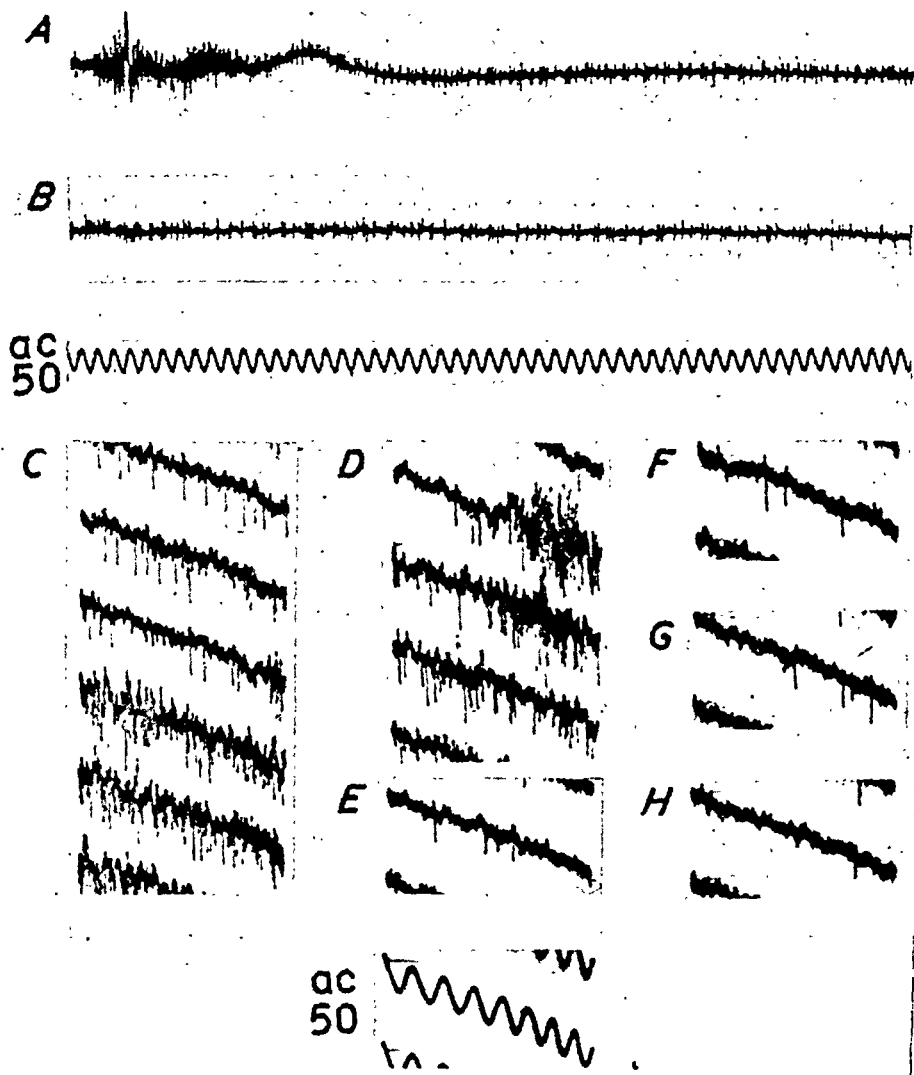


Fig. 1.

A. B. (continuous). Constant pressure. The first outburst is mainly elicited in hair touch receptors.

C. Shows the frequency of impulses elicited when light pressure (first part) as compared with heavier pressure (last part) is applied.

D—H. Shows the adaptation for constant pressure. A one minute interval between each record.

Time a. c. 50 cycles.

Further explanation in text.

On the other hand a steady pressure applied to the sural area usually gave a response of the type shown in Fig. 1, A, B. Clearly this is very slowly adapting and entirely different from the usual touch receptors. A short note on these findings has been published

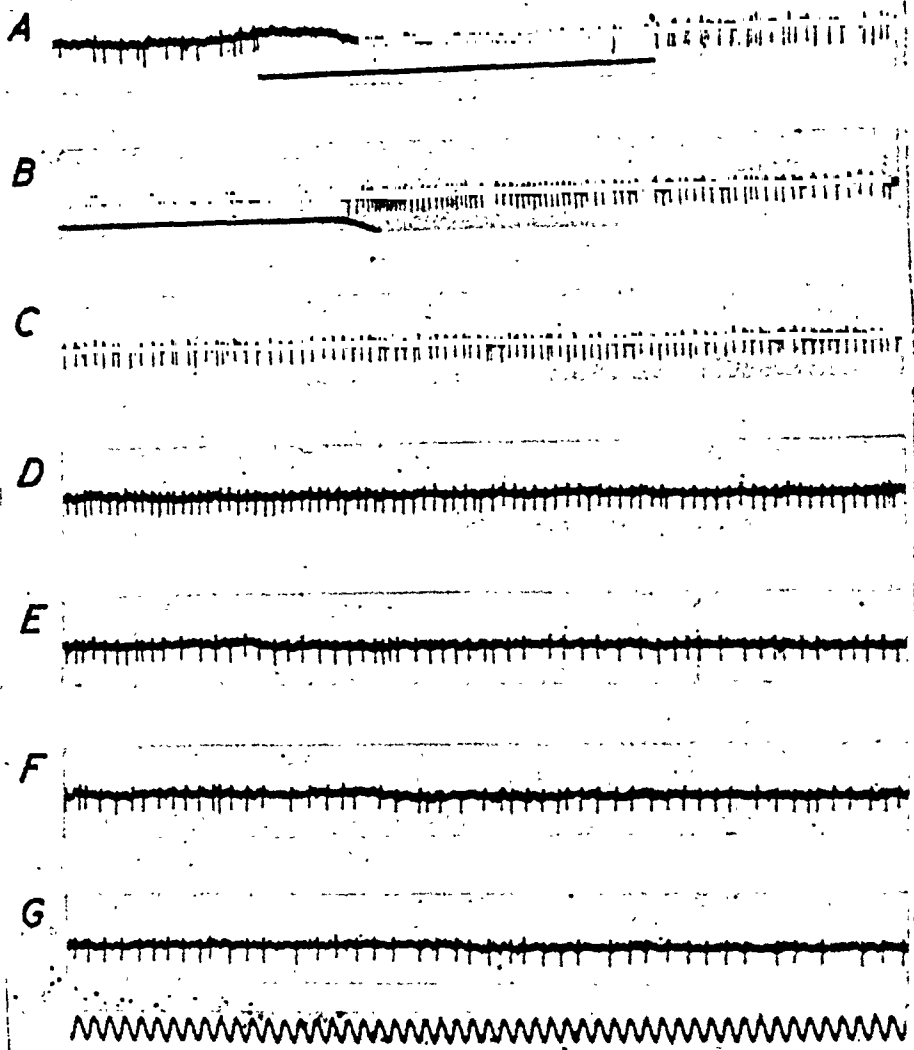


Fig. 2.

A. Pressure released for the time marked.

B—G. (continuous). Adaptation of a single "spot".

Time a. c. 50 cycles.

Further explanation in text.

in the proceedings of the Physiological Society (1948). A Pacinian corpuscle would probably give this type of response (ADRIAN and UMRATH 1929) but no Pacinian corpuscles could ever be found in this area by the histological studies (WEDDELL, GUTTMANN and GUTTMANN 1941) or in a re-examination specially directed to this matter (FRANKENHAEUSER, WEDDELL and FEINDEL 1949).

The function and the distribution of the receptor responsible for the continuous discharge was systematically studied.

The frequency of impulses elicited by a pressure on the skin in the sural area was higher for heavy than for light pressure (Fig. 1, C). For a constant pressure the frequency decreased with the time so that the discharge was extinguished after three to

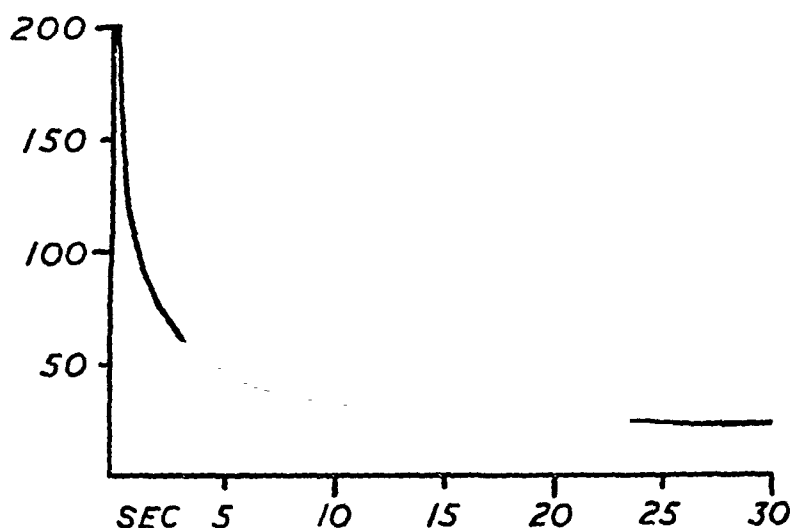


Fig. 3.

Average adaptation curve for two "spots". Frequency of impulses plotted against duration of constant pressure.

five minutes (Fig. 1, D—H). If the pressure now was released and immediately applied again a similar slowly adapting response was obtained. If the pressure was released while the discharge was continuing, impulses stopped immediately (Fig. 2, A).

Spikes of varying amplitude were recorded when a skin area of half a  $\text{cm}^2$  was stimulated, *i. e.* when several nerve fibres were activated (Fig. 1, A, B).

It should be pointed out that light movement of the hairs was not an adequate stimulus for this type of discharge. A deformation of the skin itself was necessary in order to evoke the slowly adapting response.

The sural area was systematically tested with a nylon suture thread (maximum pressure 200 mgr., 0.15 mm. diameter). A high frequency of discharge, up to about 200 per second, was elicited from a skin area of about 0.2 mm. diameter (Fig. 2, B). Such an

area is below called a sensitive "spot". At a distance of 0.2 mm. from the "spot" a low frequency (20—75 per second) was elicited by the same pressure and about 0.4 mm. from the "spot" no response whatever was obtained. Thus the receptors responsible for this type of discharge could be localized within 0.2 mm.

Some of the "spots" were marked with indian ink. They remained excitable by the nylon thread for several hours. Fig. 1, D—H, showed the slow adaptation when a large area was excited. The record contained the response of several different receptors. Fig. 2, B—G, shows the adaptation when a single sensitive "spot" is excited by the nylon thread. The discharge frequency declines over some three to five minutes, and the relation of frequency to duration of discharge is plotted in the curve shown in Fig. 3.

The uniformity of the spike heights, independent of pressure and of adaptation, are typical of a single fibre response, and in the sural area it does not appear that the 200 mgr. nylon thread ever excites more than one fibre.

Four to eight "spots" were found per cm<sup>2</sup> of the skin. They were scattered over the skin surface without any marked grouping but the distribution was usually denser in the proximal than in the distal part of the sural area. This does not necessarily mean that there are less receptors in the distal than proximal regions, but only that less of them are connected to the sural nerve (which is recorded throughout). It is in fact found that the regions of nerve overlap are those where the sural sensitive "spots" are scarce.

When folds of the loose skin were displaced across the deeper tissues the "spots" marked on them still gave the usual discharge. This shows that the receptor is localized in the skin and not in the subcutaneous tissue.

The sensitive "spots" were examined histologically and the relatively simple sensory end organ shown in Fig. 4 was found.

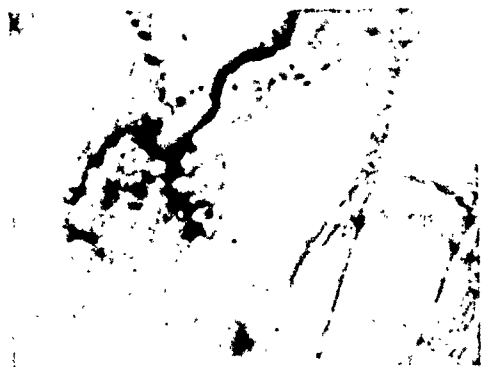


Fig. 4.

Methylene blue preparation of a marked "spot". (With permission of WEDDELL and FEINDEL.)

This work is appearing elsewhere (FRANKENHAEUSER, WEDDELL and FEINDEL 1949).

### Summary.

It was found that a pressure applied to the distribution area of the sural nerve (rabbit) elicited a slowly adapting response. The adaptation time was about five minutes. Pacinian corpuscles were excluded. The end organs responsible for this discharge were localized within 0.2 mm. Their mechanical threshold remained below 200 mgr. The discharge from a single receptor followed the all or none law. No after discharge was obtained. Four to eight receptors were found per cm<sup>2</sup> skin.

I wish to thank Doctor GRAHAM WEDDELL in whose laboratory this work was carried out.

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## Ischaemic Paralysis of a Uniform Nerve.

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### Introduction and Problem.

Recent investigations on the ischaemic blocking of nerves show some disagreement. The question of the localization of the block is the chief contradiction. Several investigators claim that ischaemia blocks the nerve first in its proximal part, whereas some claim that the most distal part of the nerve is first affected or even that the end organs might be affected at an early stage.

Most of these investigations have been carried out on humans and comparatively few animal studies have been undertaken. The animal experiments were carried out under conditions which prevent a direct comparison with the results obtained on man.

The problem of the present investigation is first to find out how different conditions in animal experiments affect the ischaemic survival time (the time up to the moment of conduction block), and then to find out which part of the nerve is first blocked. A further question which was investigated is whether there is any clear relation in the survival time for fibres conducting impulses from slowly adapting touch receptors, and rapidly adapting hair touch receptors and if such a relation can be correlated with the conduction rates in the corresponding fibres.

The technique described by ADRIAN and ZOTTERMAN (1926) to record sensory impulses from an exposed nerve was found to be a convenient basis for the experiments. The same technique has been used by ZOTTERMAN (1933) for ischaemic experiments.

A purely cutaneous nerve in the rabbit, *Nervus cutaneus surae fibularis* (below called the sural nerve), was chosen for the experiments. This nerve is small enough for leading off reasonably large single impulses in the larger myelinated fibres, and the nerve can be dissected over a relatively long stretch without branches.

### Historical Section.

It has long been known that a nerve can be kept alive outside the body for a considerable period of time provided it is prevented from drying and has access to oxygen (BOWDITCH 1890, HERZEN 1897, BRODIE and HALLIBURTON 1901, BECK 1908 etc.). If the nerve does not have access to oxygen it gradually becomes blocked (v. BAEYER 1903, BAAS 1904, GOTTSCHALK 1914, 1919, GERARD 1927, a, b, 1930, 1932, LEHMANN 1937, LORENTE DE NÓ 1947 etc.).

The oxygen consumption of a nerve has been quantitatively measured by THUNBERG (1901) and GERARD (1927 b, 1932). GERARD (1932) and later LORENTE DE NÓ (1947) have shown that an asphyxiated nerve is depolarized to a critical level at the moment when it stops conducting impulses.

The time taken to establish an asphyctic block varies for different animals and for different conditions. Thus GERARD (1930) found that an excised dog nerve becomes blocked considerably quicker than a frog nerve. A nerve with high respiration is blocked more rapidly than a nerve with low respiration. The higher the temperature the more lively is the respiration and the more rapid the blocking (within certain limits). These results have recently been verified by WRIGHT (1946) who pointed out that the survival time is shorter and the rate of oxygen uptake higher close to the cut end compared to the rest of the nerve.

So far experiments on excised nerve only have been mentioned. Several investigations have been undertaken on humans and on animals with the nerve *in situ* during ischaemia. Human experiments will now be considered, followed by animal experiments.

### Investigations on Man.

FABRITIUS and v. BERMANN (1913) investigated the loss of different sensory modalities in a finger occluded at its base by an elastic band. It was shown that the stereognostic ability dis-



appeared at a moment when touch and pressure still were felt. No sensations from touch and pressure were observed at a moment when temperature and pain sensitivity still were intact. The ability of discriminating small differences in weight disappeared simultaneously with sensation for touch and pressure. It should be mentioned that touch anaesthesia appeared after an occlusion of about 45 minutes.

LEWIS, PICKERING and ROTHSCILD (1931) carried out a systematic investigation on ischaemic blocking of human nerves and published a number of important findings. Their results on the mechanism of asphyxia were made the basis for future experiments and their technique has been used and developed by a number of later investigators (ZOTTERMAN 1933, LEWIS and POCHIN 1938, KUGELBERG 1944, 1946 a, b, WEDDELL and SINCLAIR 1947, SINCLAIR 1948, BARLOW and POCHIN 1948, GORDON 1948).

A brief account will therefore be given of LEWIS, PICKERING and ROTHSCILD's work. One or several pneumatic blood pressure cuffs were applied to different parts of the arm and the forearm for varying time periods and the sensitivity of the skin distal to the cuffs was tested. Paraesthesias appearing after application and after release of the cuff were noted and are described by LEWIS et al. It was found that the anaesthesia appears more rapidly when the cuff is applied to the proximal part of the upper limb than when it is applied to the distal part. The anaesthesia always starts in the distal part and spreads proximally. Further it was shown that different sensory modalities were blocked at different times.

It is outside the scope of the present investigation to go into further details of this paper. It ought, however, to be pointed out that the main conclusions drawn by the authors are that a nerve is more sensitive to asphyxia in its proximal than in its distal part (*i. e.* a proximo-distal gradient) and that long nerves are affected earlier than short nerves (*i. e.* a centripetal spread of anaesthesia).

These conclusions have been confirmed by most of the investigators in this field (ZOTTERMAN 1933, THOMPSON and KIMBALL 1936, KUGELBERG 1944, GROAT and KOENIG 1946 a, b, BARLOW and POCHIN 1948) but questioned by some (BENTLEY and SCHLAPP 1943, a, b, WEDDELL and SINCLAIR 1947, SINCLAIR 1948).

KUGELBERG (1944) produced nerve ischaemia by a pressure cuff and showed that all the nerve distal to the cuff exhibited an

increase in accommodation. This occurred earlier in the proximal than in the distal part of the ischaemic stretch and was also earlier in the long nerves than in the short ones.

SINCLAIR (1948) recently carried out a series of cuff experiments on man similar to those undertaken by LEWIS et al. (1931). He does not get a significant difference in blocking time when the cuff is applied high on the arm and low on the (upper) arm. Further he claims that the anaesthesia does not have a purely centripetal spread but that it affects the areas supplied by different nerves or nerve branches so that one such area becomes anaesthetic throughout its whole extension simultaneously. Hair touch sensibility is found to be blocked by ischaemia later than skin touch sensibility. He concludes that a greater sensitivity in the proximal part of the nerve is not the only reason why the nerve becomes blocked more rapidly when the cuff is applied to the arm than when it is applied to the forearm. As other influential factors he suggests that a nerve is more effectively *compressed* in the arm than in the forearm and that the blood occlusion is more effective on the arm than on the forearm. He also thinks it possible that the end organs are affected at an early stage.

THOMPSON and KIMBALL (1936) have experimented on humans with a blood pressure cuff applied above the elbow and electrical stimulation at the level of the wrist. They found that "the ischaemia of the nerve first increased and later diminished the irritability of the fibres first stimulated by a current increasing from zero". They concluded that "in mammalian nerves in vivo asphyxia affects the large fibres first". The authors investigated low threshold fibres only, *i. e.* large fibres. It seems to be mere speculation to conclude that the large fibres are affected before small fibres when the latter were never tested.

#### Animal Experiments.

Experiments on animals have been undertaken in order to study the problem under conditions more easily controlled.

It is outside the scope of this work to describe the earlier studies where a muscle twitch was used as an index for a continuous conduction of impulses in an efferent nerve since this paper deals with cutaneous nerves only.

ZOTTERMAN (1933) describes some ischaemic experiments on the cat. A rubber band was tightly tied around the thigh and impulses were led off from the exposed distal part of a sensory

nerve activated by stimulation of the paw. He concludes: "As still after 40 minutes many of the receptors and the fibres were in functional condition it is obvious that the receptors and the distal parts of the fibres either need very little oxygen or can work on a very high oxygen debt." With this finding and with his results obtained in experiments on humans ZOTTERMAN supports LEWIS, PICKERING and ROTHSCHILD's view that a nerve is more sensitive to asphyxia in its proximal part.

GROAT and KOENIG (1946 a) investigated nerve excitability after the death of experimental animals (cat). The trachea was clamped and the action potentials elicited by electrical stimulation of different parts of the exposed nerve were recorded. They concluded that an ischaemic nerve neither becomes blocked according to the principle of punctate blocks appearing in random order along the entire length of the nerve, nor is it a compact block beginning proximally and spreading distally. It is essentially the proximal part which is first affected except for some fibres where the block starts more distally and the proximal part remains intact.

BENTLEY and SCHLAPP (1943 a) in electro-physiological experiments on the cat found that in "a limb rendered ischaemic by a complete tourniquet at its root the nerve in the leg and foot becomes inactive in about 30 minutes though the nerve in the thigh survives for at least two hours". They also showed that the time difference did not depend upon a difference in sensitivity to ischaemia in the proximal and distal part of the nerve, but that owing to some access to oxygen conduction persisted longer in the thigh than in the leg.

The results obtained by all the other investigators discussed are in general agreement with each other even if there are minor points of disagreement which have led the various investigators to contradictory conclusions. But they are in complete disagreement with the results obtained by BENTLEY and SCHLAPP.

A point of considerable interest is whether the pressure of the cuff on the nerve is one of the factors responsible for paralysis. Most of the investigators take the view that the effect of pressure cannot be neglected but the extent of this effect remains obscure (ZOTTERMAN 1933, LEWIS, PICKERING and ROTHSCHILD 1931). BENTLEY and SCHLAPP (1943 a) conclude that pressure does not shorten the ischaemic survival time in tourniquet experiments.

CLARK, HUGHES and GASSER (1935) investigated the electro-neurogram during ischaemic conditions. They compared the blocking time of A, B and C fibres with the events in ischaemic experiments on humans. Later, GASSER (1943) investigated the blocking of different fibre groups in more detail. He states that "When a nerve is asphyxiated in an experimental animal, the first fibers to be blocked are those in the delta elevation; then as the asphyxia progresses, the larger medullated fibers are included. In general, the larger the fiber the longer it survives, but the size-order is not rigidly followed. At all stages of the asphyxia some fibers larger than those in which the major portion of the blocking is occurring are losing their power of conduction."

### Impulses in a Cutaneous Nerve.

It is a well known fact that impulses in an exposed cutaneous nerve can be recorded when stroking the hairs in its distribution area (ADRIAN and ZOTTERMAN 1926, ZOTTERMAN 1933, 1937, 1939, a, b, 1941). Recently it was shown that impulses from a slowly adapting receptor in the sural area in the rabbit can be recorded separately from the hair touch impulses (FRANKENHAEUSER 1948, 1949).

### Conduction Rates.

The work of GASSER et al. in correlating conduction rate, fibre size and impulse amplitude is a classic (GASSER and ERLANGER 1927, GASSER and GRUNDFEST 1939, HURSH 1939, GASSER 1943). It is outside the scope of this paper to give a complete review of these investigations but the main results should be quoted. "The velocities in A fibers depend upon their size. For all practical purposes the velocity can be described by a constant times the axon diameter, though more accurately the function connecting the two variables is probably one that would yield a flat curve approaching a straight line from a direction indicating a power slightly less than one." (GASSER 1943.)

SANDERS and WHITTERIDGE (1946) have shown on regenerating nerves that the conduction rate is more closely related to the myelin thickness than to the axon diameter. ZOTTERMAN in 1936 called attention to the influence of myelin thickness:

ZOTTERMAN (1933, 1937, 1939 a, b, 1941) measured the conduction rates for impulses in single fibres elicited by different kinds of cutaneous stimuli. He finds on the cat's saphenous nerve

that "the large potentials produced by deformation of the skin and movements of the hairs are conducted at 30—60 m/sec.". — "When the hairs are touched very lightly by moving the finest wisp of cotton wool over them the records show only  $\delta_2$  (8—17 m/sec.) potentials, often followed by a weak after discharge of C potentials." (1939 a.)

### Technique.

The rabbit was kept under urethane narcosis which was supplemented by ether inhalation when necessary. For leading off impulses, the sural nerve was exposed at the middle of the thigh or about three cm. below the knee<sup>1</sup> or at both these places. The exposed part of the nerve was kept in moist air or in liquid paraffin in order to prevent drying.

The experiments (when not otherwise stated) were conducted with the animal or the leg in free air and in ordinary room temperature, 22—23°. In some amputation experiments the leg was immediately after the amputation put in a thermostat-controlled liquid paraffin bath. The temperature of the bath was kept at either  $37.5^\circ \pm 0.5^\circ$  or at  $22.5^\circ \pm 0.5^\circ$ . The temperature in the proximal and distal part of the leg was in a series of experiments observed by the use of iron-constantan thermocouples connected to a Cambridge spot galvanometer.

Silver electrodes were used for the leading off, and a two-channel condenser coupled amplifier, cathode ray tubes and a loudspeaker were used for recording the impulses. Photographs were occasionally taken on bromide paper.

### Methods of Making the Leg Ischaemic.

In one group of experiments the trachea was clamped in order to make the whole animal ischaemic. The trachea was first prepared and when the nerve had been exposed, the trachea was occluded with artery forceps. The onset of ischaemia was measured from the moment of clamping.

In some experiments the leg was amputated at the two levels mentioned.

In another group of experiments a "sphygmomanometer cuff" was applied around the leg. The cuff consisted of two thin walled rubber tubes surrounded by silk on a wooden clamp. The width of the cuff was 4 cm. The pressure in the cuff was kept at 150 mm. Hg. The cuff was applied at different levels of the leg and its position was marked by its upper border on the leg. A 4 cm. elastic rubber band, tightly tied around the leg was used alternatively with the sphygmomanometer cuff.

<sup>1</sup> The electrodes at the middle of the thigh are below for simplicity called the proximal electrodes and the electrodes below the knee the distal electrodes. Unless otherwise stated the expression "at the thigh" means at the *middle* of the thigh and "below the knee" means about *three cm. below* the knee.

### Stimulation.

A stroke over the hairs or a pressure applied to the skin were used as stimuli. The strength of these stimuli varied considerably from time to time but they were nevertheless found suitable since they made it possible to record the survival time of even the last blocked fibres, whereas a more localized stimulus activates a few fibres only and may leave others conducting.

### The Survival Time.

By "ischaemic survival time" is here meant the interval between the moment of circulatory arrest (or of trachea clamping) and the moment when no impulses can be recorded from the nerve upon stroking the hairs. When instead of hairs the slowly adapting end organs are stimulated in order to measure the survival time of the corresponding fibres, this is always mentioned. The term "ischaemic survival time" is used for convenience and the question of whether this time is affected by a pressure to the nerve or other causes, is at the moment left open. The main thing is to be sure that the conduction block is not complicated by other factors affecting the *exposed* part of the nerve, *e. g.* drying, mechanical injury etc.

The error for the determination of the survival time was judged not to exceed two minutes. The moment of onset of ischaemia could be determined within  $\frac{1}{4}$  of a minute and the moment of complete blocking within  $1\frac{1}{2}$  minutes.

Several experimental errors were reduced by performing two experiments simultaneously (within 1—2 minutes) on both limbs of an animal. Sometimes the two experiments were identical and provided a check upon random variation; sometimes the circulatory arrest or amputation was performed at different levels in the two limbs and afforded a control against variation in narcosis or body temperature since this should affect both limbs similarly.

## Results.

### Preliminary Controls.

The majority of the experiments in this paper are concerned with the circulatory arrest in nerve and the measurement of the time this condition must persist before it produces a conduction block.

Now there are several factors which could conceivably stop impulses being recorded. Before an ischaemic conduction block or an ischaemic end organ paralysis can be investigated it must be ascertained that the nerve is not blocked at the recording site and that excessive fluid does not short circuit the electrodes.

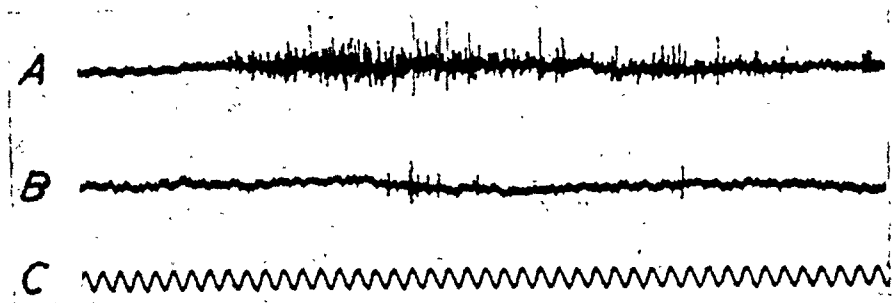


Fig. 1. Typical action potentials elicited by a stroke over the hairs during an ischaemic experiment. Record A: before ischaemia, record B: a few minutes before complete ischaemic block.

Conduction block was proved to occur in the ischaemic part of the nerve within the leg and not in the exposed part, because when the block had appeared, squeezing the nerve at the place just peripheral to where it became exposed, still gave recorded impulses. Moreover, when the circulatory arrest was released, the impulses were conducted normally again within a minute or two. This is clearly inconsistent with the interpretation that "block" was due to drying or short circuiting at the recording site. In fact the degree of shunting at the electrodes was always controlled throughout the experiment by observing the size of the spike recorded from a uniform group of fibres, and keeping this constant, as ZOTTERMAN has described (1939).

In several experiments a considerable motor activity was noted. This interfered with the recording of sensory impulses. It was found that a few drops of ether in the neighbourhood of the rabbit's head depressed this activity. These findings have been described in more detail (FRANKENHAEUSER and LUNDERVOLD 1949). Such use of small quantities of ether, 2—3 drops, was found a convenient method of depressing the motor activity whenever needed during the experiments.

#### Proximo-distal Gradient.

In these experiments the trachea was clamped and the ischaemic survival time recorded (Fig. 1). When the nerves in both legs of the animal were simultaneously exposed at equal levels, the survival time was found to be equal. When the impulses were led off from the thigh<sup>1</sup> in both limbs, both were blocked at 26 minutes

<sup>1</sup> See foot note p. 7.

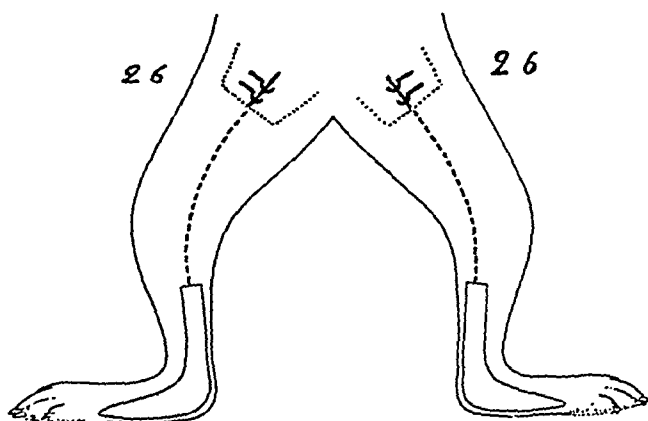


Fig. 2. In this and subsequent figures the representation is as follows: The sural area is indicated by the bootshaped figure. The line representing the sural nerve is interrupted in that part where the nerve lies buried within the leg. The electrodes are shown by hooks, the incision by a dotted line, and the survival time (minutes) by the figure on the corresponding side.

Fig. 2. Trachea clamp experiment. Operation in the middle of the thigh.

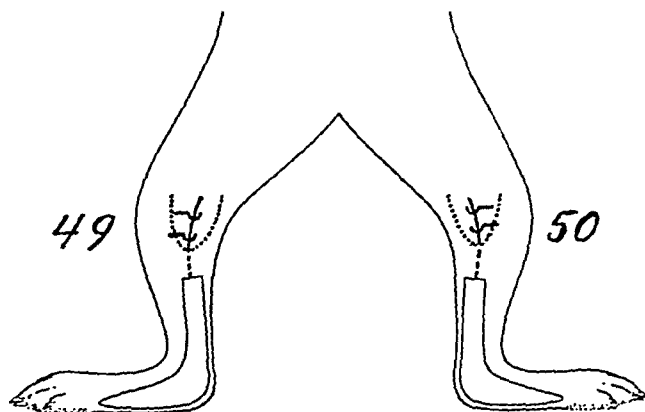


Fig. 3. Trachea clamp experiment. Operation below the knee.

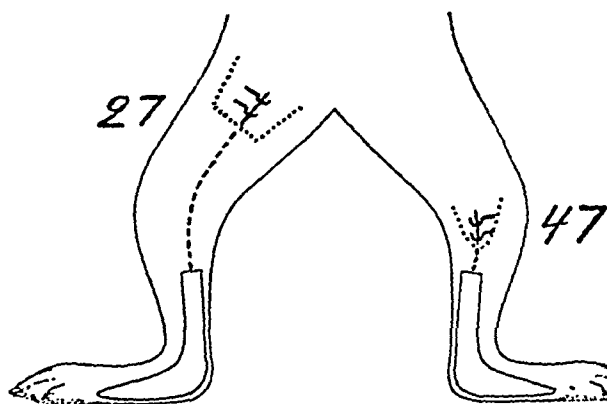


Fig. 4. Trachea clamp experiment. Operation in the thigh and below the knee.



(Fig. 2), when from below the knee, the survival times were 49 and 50 minutes (Fig. 3). When one nerve was exposed in the thigh and the other below the knee (Fig. 4), the survival times were 27 and 47 minutes respectively as observed from records taken simul-

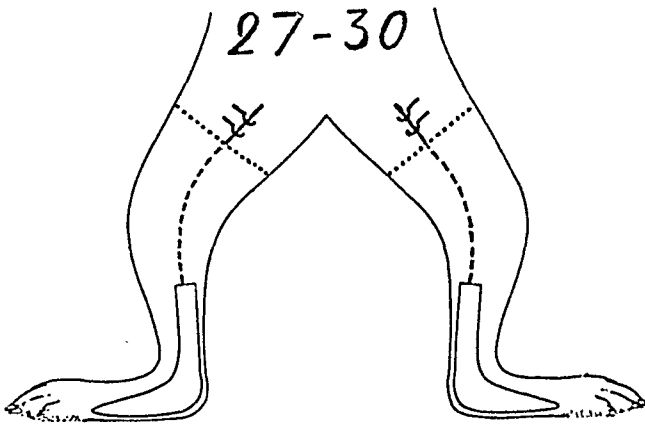


Fig. 5. Amputation experiment. The numbers indicate the extreme values of survival times obtained in several experiments.

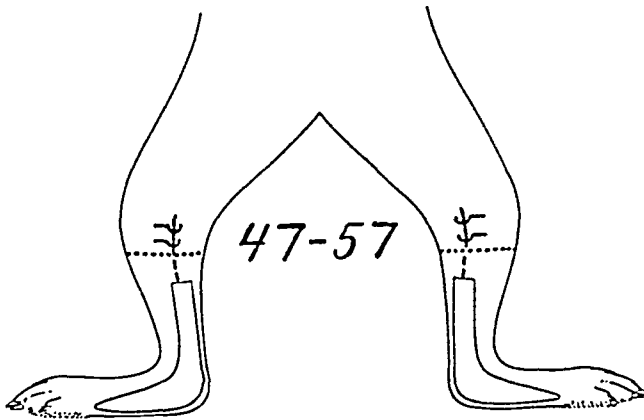


Fig. 6. Amputation experiment. The numbers indicate extreme values from several experiments.

taneously. It is thus clear that the nerve is blocked some 20 minutes earlier at the middle of the thigh than below the knee.

Now when the trachea is clamped the circulation continues for a short time and could bring to the nerve toxic products resulting from ischaemia elsewhere in the body. Such effects will not arise in ischaemia of the leg resulting from amputation.

The sural nerve was exposed either in the thigh (Fig. 5) or below the knee (Fig. 6), sometimes at the same level on each leg and sometimes different. The legs were amputated just above the exposed nerve in each case, and the survival times measured.

The extreme values found were 27—30 minutes in the thigh and 47—57 minutes below the knee, and these lie so close to the values (above) when the trachea was clamped, that there are no grounds for supposing circulating toxins to be involved in the nerve block.

Now the difference in survival time in the two regions of the leg might be due to some difference in susceptibility inherent in the nerve in the two regions, or it might be due to differences in the nerve environment, principally temperature gradient and oxygen leakage. This question was investigated as follows.

### The Influence of Temperature.

The nerves were exposed in the thigh and the legs were amputated just above the exposure. After amputation, the legs were put in liquid paraffin baths of either 37—38° or 22—23° temperature, and the survival times were measured in the usual way. At 37—38° the mean survival time was 18.5 minutes and at 22—23° temperature the value was increased to 34 minutes. This gives a difference of 1.1 minutes in survival time for an alteration of one degree in temperature. Thus the temperature ought to be about 18° lower at the distal part of the leg than it is in the proximal to account for the 20 minutes difference in survival time observed in the former set of experiments. Now it is quite evident that such a temperature gradient does not exist in the leg, but on the other hand it is very likely that the calculated coefficient is too low, since the temperature in the leg does not equilibrate at once with the bath temperature. So, though the bath temperature varied by 15° the leg temperature changed by some smaller amount. It is unlikely that this could be less than 7.5° so that the real coefficient should be less than 2.2 minutes per degree. From this follows an expected difference in temperature at the two recording sites of 9°. Now the temperatures at these two points were measured by thermo-couples and an average curve for two experiments is given in Fig. 7. This shows that five minutes after clamping the trachea, the temperature is about 1° higher in the thigh than below the knee and after 12 minutes the temperature is equalized, after this the thigh is cooler than the leg (on account of greater heat loss through the larger operation wound). A temperature difference of 1° throughout the whole experiment would give a time difference of only 2.2 minutes. This value lies within the variation between single experiments,

so the temperature gradient has a negligible influence on the surviving time. Clearly we must look elsewhere for the explanation of the 20 minutes difference found in the survival times at thigh and knee.

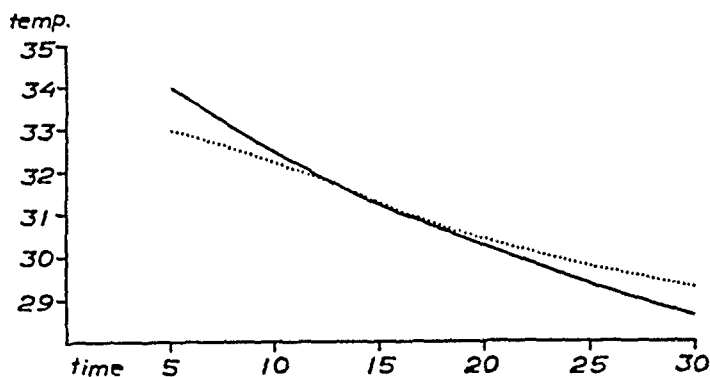


Fig. 7. The temperature in the thigh (full line) and below the knee (dotted line) plotted against time after amputating the leg (kept in air of room temperature). The curves are average curves from two experiments. Single readings deviate by less than one degree from the curves.

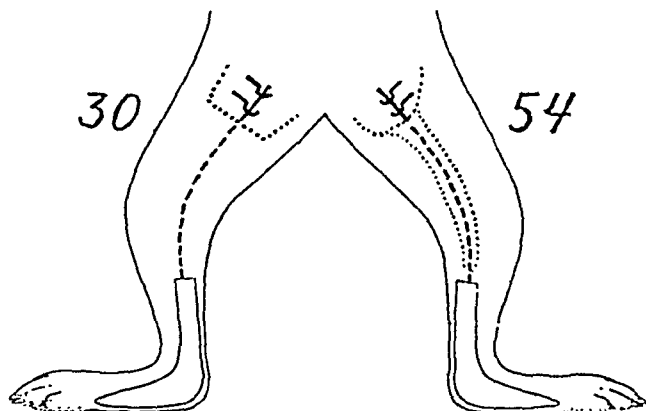


Fig. 8. Trachea clamp experiment. The skin was slit over the nerve on one leg.

### Oxygen Leakage.

The nerves rendered ischaemic by clamping the trachea were not entirely deprived of oxygen, because the recording site was exposed to air. It seemed possible, therefore, that some oxygen might diffuse into the leg about the recording site and prolong the survival time of the nerve in the neighbourhood. To test whether the skin was in fact an effective barrier to oxygen diffusion the following experiment was done (Fig. 8).

The sural nerve was exposed at the thigh on both sides. On one side the distal part of the leg was left intact. On the other, the skin over the sural nerve was opened. The subcutaneous tissue and the nerve were left untouched. The trachea was clamped and the survival times were determined. It was found that the survival time was 30 minutes in the intact leg and 54 minutes in the other.

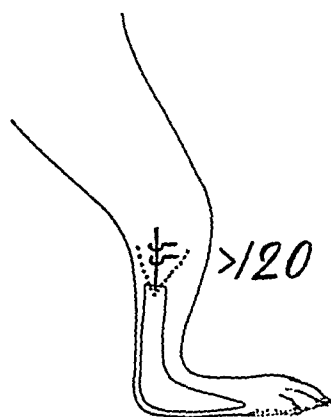


Fig. 9. Trachea clamp experiment. The incision was extended into the sural area.

In sharp distinction to all the former experiments in which similar regions of ischaemia were associated with nearly identical survival times, the present results show clearly that when merely the skin over the nerve is cut, the survival time is much prolonged. A similar result was found in some experiments where the nerve was exposed below the usual distal level so that a part of the subcutaneous tissue underlying the sural area had free access to

air (Fig. 9), the survival time was markedly longer, up to two hours. The spikes could only be elicited for this long time from the proximal part of the sural area which was rather near the skin incision.

Clearly then the skin is an important barrier to oxygen diffusion and differences in the accessibility of oxygen from operation wounds must be excluded before we can safely draw conclusions about the local susceptibility of various regions of nerve. Such information may be obtained when the circulation is arrested at various levels by means of tourniquets.

In each leg the nerve was exposed at the thigh (Fig. 10). Elastic bands were tied tightly round the legs, on one side above the knee, on the other below it. The survival time was simultaneously determined for both sides. In one experiment the survival time was 27 minutes for each leg; in another 28 minutes for each leg. These experiments show that when the entry of oxygen through the operation wound is excluded, the survival time is the same for different lengths of ischaemic nerve.

There are three possible interpretations. Either there is no gradient of susceptibility in the nerve, or the blocking in the present experiment is not due to ischaemia but to the pressure of the tourniquet, or the failure was not due to conduction at all but to ischaemic paralysis of the end organ.

Now the pressure of the tourniquet has no action in blocking conduction apart from circulatory arrest as can be seen from the following experiments. Both legs had the sural nerve exposed in the thigh (Fig. 11), and were simultaneously amputated just

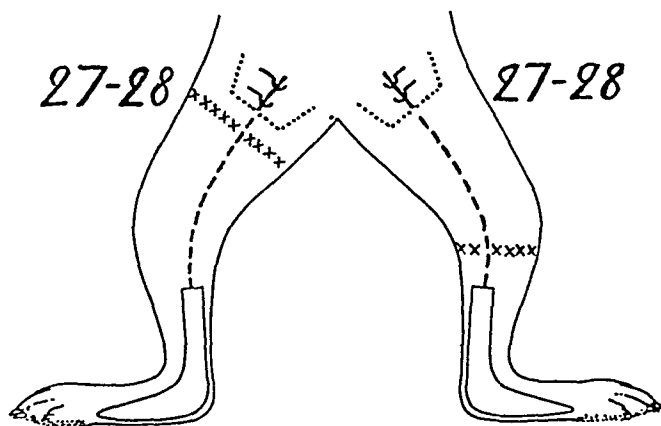


Fig. 10. Tourniquet experiment. The proximal border of the tourniquet is indicated by 000.

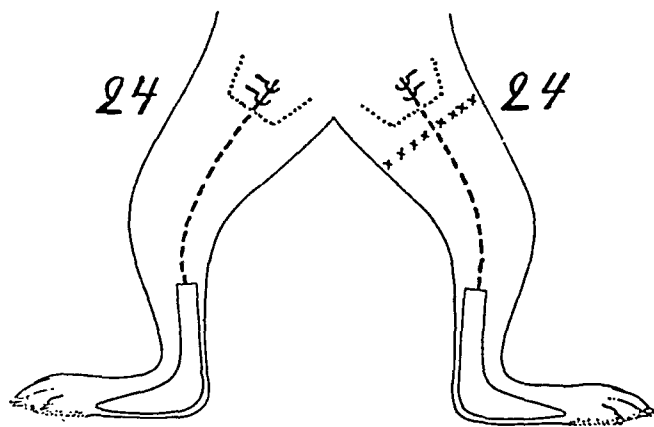


Fig. 11. Amputation experiment with a tourniquet on one leg.

above. On one leg only, an elastic band was tightly tied round the leg above the knee, and both legs were put into a bath of liquid paraffin at  $35^{\circ}$ , and the survival time measured. Both legs had the same survival time of 24 minutes. Thus the pressure of the tourniquet upon the nerve was quite insufficient to affect the survival time.

### The Survival of Ischaemic End Organs.

When impulses fail at the end of the survival time, it is of prime importance to know whether this is due to the conduction

block in the ischaemic nerve, or whether the ischaemic end organs no longer respond to stimulation as suggested by SINCLAIR (1948). The following experiment proves that the block in nerve conduction is the limiting factor.

The nerve was exposed (Fig. 12) at the thigh and also just above the sural area (note that this level is more distal than in the other experiments). The operation wound at the distal point was made as small as possible for leading off. The trachea was clamped and the survival times were determined as usual. At the proximal end the survival time was about 30 minutes, at the distal point about 80.

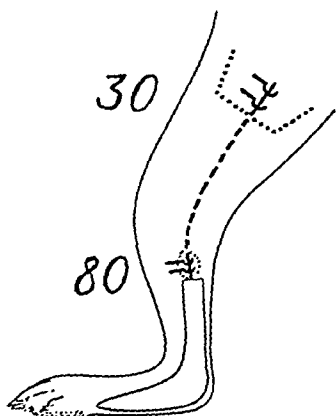


Fig. 12. Trachea clamp experiment. Nerve exposed through small distal incision.

From this it is plain that between 30 and 80 minutes from the clamping of the trachea the leg was in a condition where the end organs still responded and the impulses were seen at the distal

electrodes but did not reach the proximal ones. Moreover the survival time in this clear case of conduction block was about the same as in the former experiments when recording from the thigh.

It is not unlikely that the end organ in the present experiment received a little oxygen from the small operation wound. So it is not claimed that the ischaemia of the end organ in this case is the same as in the former experiments. But it is claimed that inexcitability of the end organ could not be the limiting factor in the other experiments since it clearly was not in the present one which had the same survival time.

The main argument from the foregoing experiments may now be summarized. The survival time of a short stretch of nerve, is always longer than that of a great stretch of nerve, whether ischaemia is produced by amputation or by clamping the trachea. This is probably due to oxygen being able to diffuse in from the skin incision to all parts of the short stretch, for when the skin was slit over the long stretch, its survival time was prolonged to the same value. In tourniquet experiments oxygen diffusion cannot take place, and no difference is found in the survival time of the long and the short ischaemic stretches of nerve, since this equality is due neither to the pressure block of the tourniquet

nor to an early failure of the end organ to respond, it must be due to an equal susceptibility of the two regions of the nerve. The view that there is a regular gradient of ischaemia-sensitivity down the nerve is therefore disproved.

#### A Comparison of the Survival Time in Fibres from Hair-Touch and Slowly Adapting Receptors.

It has been shown that impulses in nerve fibres from slowly adapting receptors can easily be recorded separately from the impulses in fibres from hair touch receptors (FRANKENHAEUSER 1949). Since the largest spikes are of about equal amplitude in both these groups of fibres it is of interest to investigate the difference in survival time between them. The two survival times were measured in most of the experiments described. It was regularly found that the fibres conducting impulses from slowly adapting receptors were blocked later. This time difference varied from 5—15 minutes. This relation in survival time between the two different types of fibres was the same regardless of the method used for rendering the leg ischaemic, and was not altered even when the tourniquet applied a considerable pressure to the nerve.

From these experiments it cannot be determined if every fibre conducting impulses from slowly adapting receptors is intact by the time when all fibres from hair touch receptors are blocked. It is nevertheless clear that the majority of these fibres still are conducting at this moment.

#### Conduction Rates.

*Technique.* The conduction rates in single fibres were measured using a technique earlier described by ZOTTERMAN (1939). The sural nerve was exposed as close to the sural area as possible and as high on the thigh as possible. From these two parts impulses were led off and recorded with two amplifiers on a double beam cathode ray tube. Inspection of the records taken simultaneously on moving paper showed the same pattern of impulse discharges in each, but those from the proximal leads arrived later by a fixed interval due to increased conduction time. The conduction velocity was obtained by dividing this interval into the length of nerve between the two sets of electrodes, measured at the end of the experiment. Hair touch receptors and slowly adapting receptors were stimulated separately, and so it was possible to measure in the same experiment the conduction rates in single fibres of these two different types.

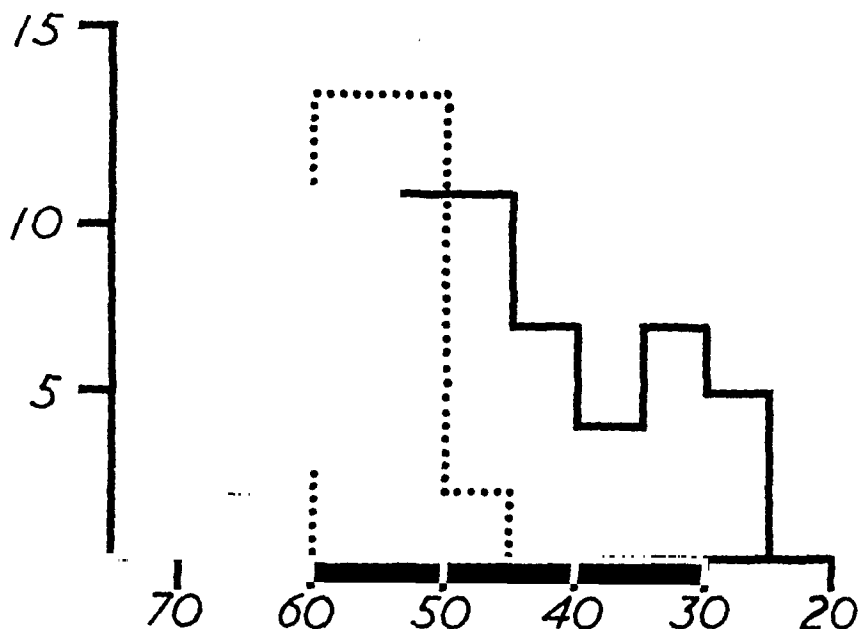


Fig. 13. Frequency distribution curves of "hair touch fibres" (full line) and "slowly adapting fibres" (dotted) plotted against conduction rate (abscissae). The ordinates show the number of fibres measured in each velocity group. The numbers for "hair touch fibres" slower than 45 m/sec. are too low because of difficulties in identifying these small impulses in the records.

### Results.

Since the hair touch receptors are supplied by fibres of widely varying size (ZOTTERMAN 1939) and the slowly adapting receptors are represented by a fairly narrow size group, the investigation aimed to find the extent of overlap between the fibre groups. Less attention was paid to the absolute conduction rates. In order to exclude experimental errors, such as differences in temperature in different experiments and the relatively large unavoidable error in measuring the length of a nerve of the size of the sural nerve, a comparison between the two fibre groups was always made within the same experiment.

It was regularly found (Fig. 13) that some fibres conducting hair touch impulses have a conduction rate higher than that of all the investigated fibres from slowly adapting receptors in the same preparation, though it was quite evident that most of the fibres conducting hair touch impulses have a conduction rate which is substantially slower. It is thus obvious that hair touch fibres completely overlap the main bulk of fibres from slowly adapting receptors.



This is interesting because, as we have seen, in ischaemia the main bulk of fibres conducting impulses from slowly adapting receptors still conducted at a moment when every fibre conducting impulses from hair touch receptors was blocked.

### Discussion.

The results obtained in the present investigation will now be compared with those obtained by previous investigators.

The difference in survival times seen when the nerve was exposed at different levels in the leg is shown to be due to relative accessibility to atmospheric oxygen. This factor does not seem to have been taken into account in the previous investigations on ischaemia in animals.

ZOTTERMAN (1933) during ischaemia led off impulses from the exposed distal part of a nerve, and found that this was still conducting after 40 minutes. He concluded that the receptors and the distal part of the nerve need very little oxygen. This conclusion is confirmed by the present results. He further stated that these experiments confirm LEWIS et al's view that the nerve itself is more sensitive to ischaemia in its proximal part. Since, however, the distal part of the nerve had access to external oxygen (skin cut through) this fact must be taken into account (p. 014). In addition to this, inhomogeneities may cause a smaller sensitivity of the distal part of nerve (p. 021).

Since it has been established that the skin is an oxygen barrier, it is evident that the proximo-distal gradient obtained by GROAT and KOENIG (1946 a) on an exposed nerve does not necessarily apply to a nerve inside an intact ischaemic limb.

The sural nerve is relatively homogeneous without any visible branches, in this nerve no proximo-distal gradient in the nerve itself was found. This shows that a steady gradient is not a regular feature of nerve, but evidently non-uniform conditions such as the transition from root to trunk or the subsequent branching of the nerve might be associated with changes in susceptibility to ischaemia. The present work has sought to eliminate such non-uniform causes of susceptibility change, and the conclusions do not contradict other work such as GROAT and KOENIG (1946 b) where greater anatomical heterogeneity contributed to the results.

On the basis of the present investigation it may be understood why BENTLEY and SCHLAPP (1943 a) obtained results contradictory to those obtained by all other investigators. BENTLEY and SCHLAPP used a very extensive operation method: the skin was cut through and sutured, the nerve was in most experiments dissected free throughout its whole length. From this it is evident that it is extremely difficult to judge the oxygen leakage at different levels on the leg. It was also shown by BENTLEY and SCHLAPP that the long survival time at the thigh was due to access to oxygen. They did not show, however, that the experimental manipulations were the reason for an oxygen leakage. Such a leakage must have affected their results profoundly.

Accurate experiments on excised nerve would obviously throw valuable light on many of the problems involved in the present paper. It was disappointing therefore to find that previous work (WRIGHT 1946) exhibited such variability that sharp conclusions could hardly be drawn. Nevertheless it appeared possible that a strict control of experimental conditions might overcome these difficulties and so the experiments were attempted. The excised nerve was bent double, stimulated at the bend and recorded from the two free ends separately. The intervening region of doubled nerve was passed through an oxygen-free chamber. But though the two stretches were in this way subjected to an environment which must have been nearly identical, the survival times proved most erratic, and sometimes the proximal and sometimes the distal survived twice as long as the other. As these experiments only confirm the variability found in former work, it is not worth describing them in any further detail.

Turning to experiments on man, the clearest evidence that we possess about the ischaemic condition of various points on the nerve are the results of KUGELBERG (1944), for he stimulated the nerve through the skin at two points and compared the sensory and motor thresholds and the accommodation rate at various stages of circulatory arrest and release. These results showed very clearly that changes occurred earlier proximally than distally.

KUGELBERG's results were obtained by threshold and accommodation measurements, and hence signify that the distal electrode encounters fibres or parts of fibres which are more resistant to ischaemic changes. This is in sharp conflict with the results on the sural nerve where no such increased resistance was found. The explanation appears to lie in the uniformity of the sural

nerve on the stretch examined. Most nerves of the limb through branching alter their gross size, and many other details in the anatomy of the nerve, sheaths and vascularization. One is tempted to suggest that these non-uniformities on an average increase the nerve resistance to ischaemic change. This idea is consistent with the observations of LEWIS et al. (1931), ZOTTERMAN (1933) etc., and it modifies their interpretation only in so far as the proximo-distal gradient is not uniform (as they supposed) but seems to depend upon non-uniformities.

We may dismiss two of the three alternative suggestions proposed by SINCLAIR. The nerve is not blocked by the *pressure* of the tourniquet as was shown by LEWIS et al. (1931) and by ZOTTERMAN (1933) and by BENTLEY and SCHLAPP (1943 a, b) and by the experiment described here on p. 015. The end organs are not paralysed by the time nerve conduction is fully blocked, as proved on p. 015. As to SINCLAIR's third suggestion that appreciable blood enters beneath a distal pressure cuff, I can only say that this does not occur in the rabbit.

The present work does not confirm SINCLAIR's observation that the whole sensory area of a single nerve branch is paralysed at the same time. For it was regularly found that in the sural area, the proximal part continues to respond some 3—7 minutes longer than the distal, in support of the views of LEWIS et al (1931), ZOTTERMAN (1933), KUGELBERG (1944).

The simultaneous observation of impulses from hairs and slowly adapting receptors brings out a fact of considerable interest. Both kinds of impulse have about the same spike height, and thus the two types of fibres are of about the same size. But with regard to conduction velocity, there is much more variation in the fibres from hairs than in the other group, and some "hair fibres" can be found which conduct faster and a great many which conduct slower than any observed fibre from the slowly adapting receptors. We might therefore expect the latter fibres to be blocked by ischaemia at some time between the earliest and the latest "hair fibres". But actually, as we have seen (p. 017) they still conduct when *all* the hair fibres are blocked. It follows that not only are these fibres connected to a different type of end organ, but the fibres themselves have different properties that cannot be predicted by simply observing the impulses.

The estimate of fibre size from the ischaemic survival time (LEWIS, PICKERING and ROTHSCHILD 1931, ZOTTERMAN 1933,

LEWIS and POCHIN 1938) has already been shown to be somewhat unreliable (GASSER 1943). The present work, confirming GASSER, may point to an interesting correlation. It is possible that the minority fibre groups which are blocked out of turn do not merely represent a random variation in the population. In the case investigated here, the late blocked fibres belong to endings with a quite special function and it may well be that this is an example of a general property. Perhaps GASSER's minority groups also subserve distinct modalities of sensory function.

### Summary.

The ischaemic blocking of a sensory nerve (the sural nerve) in the rabbit was investigated. The survival time was determined by recording sensory impulses elicited by stimulating skin receptors.

In amputation and trachea clamp experiments the survival time was longer in short stretches of ischaemic nerve than in long stretches. This difference was shown to be due to oxygen leakage. In tourniquet experiments, where oxygen leakage was prevented, long and short stretches survived for equal times. Thus no gradient in sensitivity to ischaemia in the sural nerve was found. The questions of temperature gradient in the leg, of pressure of the tourniquet upon the nerve and of end organ paralysis were all investigated and shown to be without effect upon ischaemic paralysis in the conditions of these experiments.

In spite of the fact that no gradient in ischaemia-sensitivity in the nerve was found, the proximal part of the sural area responded some 3—7 minutes longer than the distal.

Fibres from slowly adapting receptors were blocked later than fibres from hair touch receptors, although the conduction rates of "hair touch fibres" were shown to overlap completely those of "slowly adapting fibres".

The findings in the present paper are compared with earlier investigations.

This work was started in the Department of Human Anatomy, Oxford, at the suggestion of Doctor WEDDELL and arose out of the ideas of Doctor SINCLAIR. It was completed at the Nobel Institute for Neurophysiology, Stockholm.

I wish to express my sincere gratitude to Doctor GRAHAM WEDDELL, Oxford, and Professor RAGNAR GRANIT, Stockholm, for the interest they have shown in my work and for the facilities they have given me in their laboratories.

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## Ascorbic Acid Synthesis in Rats on Various Diets.

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If rats are placed on a diet containing no ascorbic acid, no symptoms of deficiency result, even if the experiments are extended over several generations (SVIRBELEY 1933 and SUDEN and ALLEY 1935). Thus rats are independent of a supply of ascorbic acid in the diet and investigations by, int. al. SCHEUNERT and SCHIEBLICH (1937), in which guinea-pigs could be kept free of scurvy by supplying rat-liver, show that an ascorbic acid synthesis takes place in the rat organism. This ascorbic synthesis has been the subject of comparatively extensive investigation. We shall only mention here that SVIRBELEY (1936) found that rats, in order to obtain a normal tissue concentration of ascorbic acid must be supplied with vitamins belonging to the B-group. In agreement with this SURE et al. (1939) found that the content of ascorbic acid in kidneys and liver decreased on a deficit of aneurin with 20—30 %, and on a deficit of riboflavine with 40—50 %. RAY et al. (1946) found that an increased excretion of ascorbic acid after administration of chloretone only appeared when the animals received sufficient aneurin in the diet. The significance of a sufficient supply of protein has been discussed by *e. g.* HOPKINS et al. (1935), whereas, on the other hand, HEINEMANN (1936) considers that the differences observed in the excretion of reducing compounds on varying supplies of proteins are not conditioned by ascorbic acid but by other substances. On the other hand,

ROBERTS and SPIEGEL (1946) state that rats, which obtain a very low supply of protein show a sharp increase of ascorbic acid excretion, if they receive methionine or cystine.

JONSON, OBEL and SJÖBERG (1942) place ascorbic acid synthesis in rats in connection with the supply of A-vitamin through the diet. On the one hand, there are great likenesses between the pathological-anatomical lesions in musculature and teeth at A-deficit and on restricted supply of ascorbic acid and, on the other hand, the ascorbic acid serum values gradually sink to zero, when the A-vitamin is withdrawn from the diet. According to PHILLIPS (1940) cattle, also, are dependent for their ascorbic acid synthesis on a sufficient supply of A-vitamin. This, however, according to RUBIN and BIRD (1943), is not the case with chickens.

In a great number of experiments (for references to the literature see EKMAN 1944) it has been found that rats, receiving normal diet and different cyclic compounds in addition, react with a sharp increase of their ascorbic acid excretion. In this connection it should be pointed out that LONGENECKER et al. (1940) by biological tests on guinea-pigs were able to show that the reducing compounds, which were excreted in great quantities, had a protective effect against scurvy and thus genuinely consisted of ascorbic acid. In an earlier paper (EKMAN and STRÖMBECK 1947) it was shown that only in rats receiving a complete diet with an adequate supply of B-vitamin were found sharply increased ascorbic acid values in the urine on 2,3-azotoluene being administered. In one of the experiments it was shown that if the rats, in addition to a diet principally consisting of rice flour and small quantities of carrot ("rice diet") received an addition of only riboflavine, their ascorbic acid excretion increased from about  $\frac{1}{2}$  mg. to over 4 mg. per day.

The excretion of ascorbic acid after the administration of cyclic compounds has been generally interpreted as an attempt of the organism to detoxicate the applied compound with the help of ascorbic acid. In agreement with this we considered that, when we had found (EKMAN and STRÖMBECK 1947) simultaneously with variations in the ascorbic acid excretion changes in the excretion of some of the split products of azotoluene, m-toluidine, m-amino-benzoic acid and aminocresol, these were secondary to the influence of the diet variations on the ascorbic acid synthesis. The aim of the present investigation is partly to examine more closely which factors in the diet are responsible for the variations in the



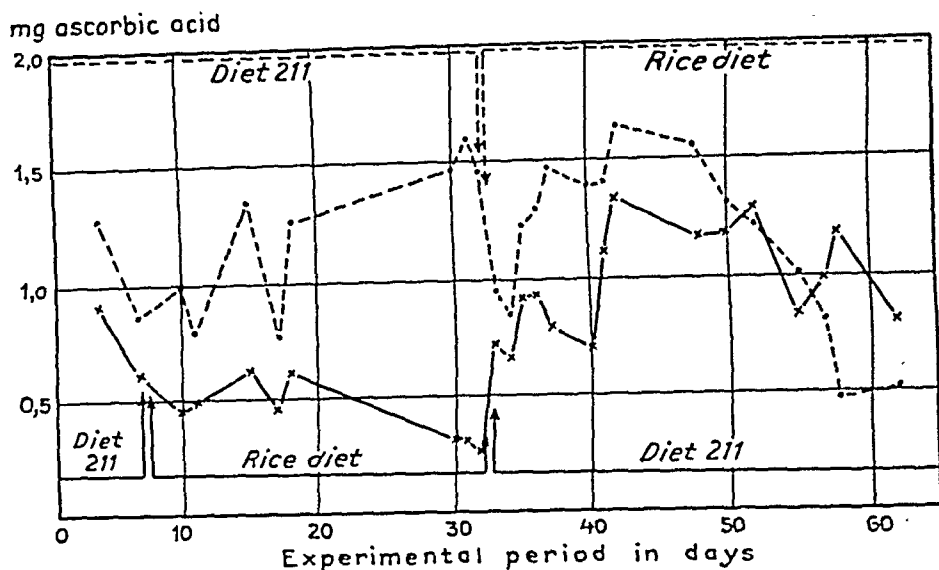


Fig. 1. Output of ascorbic acid in the urine of rats on various diets. Each curve corresponds to the average values for 5 animals.

ascorbic acid excretion, and partly to investigate whether a secondary ascorbic acid deficiency at, for instance, riboflavin deficit in the diet influences the deficit symptoms which then arise.

### I. Sufficient administration of riboflavin and casein is necessary for the normal ascorbic acid synthesis.

First, we checked our earlier results, obtained with experiments on rats, which had received azotoluene, to ascertain whether they were also applicable to animals which had not received any aromatic compound. In these and following experiments 24-hour quantities of urine were collected in flasks, containing 5–10 cc. toluene together with 10 cc. 20 % metaphosphoric acid. The determination took place through titration with Tillman's reagent (see further EKMAN 1944).

Fig. 1 shows that also in rats, which have only received either a complete diet (HAMMARSTEN's (1937) diet no. 211) or the previously mentioned rice diet, similar changes appeared. The excretion of ascorbic acid lay at around 1 to 1 1/2 mg. on the complete diet and decreased with the change over to the rice diet to around 1/2 mg. The diets differ from one another mainly by the low protein and B-vitamin content in the rice diet. Fig. 2 illustrates an experiment to analyze more closely the effect of the individual factors. The ascorbic acid excretion is compared in two groups of animals, each one containing six rats, and each receiving a diet

mg ascorbic acid

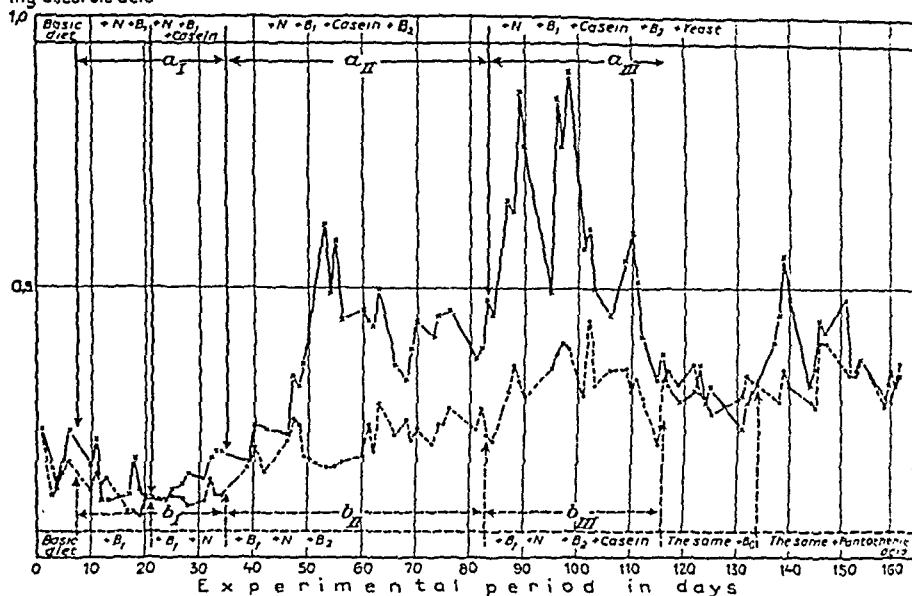


Fig. 2. Output of ascorbic acid in the urine of rats on a fully controlled diet with varying additions of vitamins and casein. Each curve corresponds to the average values for 6 animals.

fully controlled as regards proteins and vitamins: the ingredients being 88 % wheat starch, 5.7 % peanut oil, 0.3 % cod liver oil and 6 % salt mixture. In this "basic diet" part of the wheat starch was then replaced by casein up to 23 % of the mixture and different vitamin mixtures. After both groups had shown the same excretion values during some days on basic diet, an addition of 0.5 mg. niacin and 0.1 mg. aneurin per day during a first period was given to group A, but only aneurin to group B. In a succeeding period niacin, aneurin and casein (23 % of the total diet) was given to group A, and to group B only aneurin and niacin. Neither in comparison with the initial period or in comparison between the groups do any differences in the ascorbic acid excretion appear. At a later period a further addition of 0.1 mg. riboflavine per day was given to both groups, which thus are now differentiated by group A alone receiving casein. In both groups is found an increased excretion of ascorbic acid, which, however, is considerably higher in the group receiving casein. A further increase of ascorbic acid excretion was obtained in the casein-group (A) by administering ca. 1 g. yeast every day, and in group B by the administration of casein. The experiment was concluded by administering 0.1 mg. adermin and later 0.1 mg. pantothenic acid every day to group B without any variation in the ascorbic acid

excretion making its appearance. The variations in the ascorbic acid excretion now described appear, of course, clearly in the diagram but the figure material has, moreover, been statistically investigated. It was then shown that the increase at the different additions of casein or riboflavine to the different groups was statistically established both on comparison between the groups and on comparison between the different investigation periods within each group. (Thus both the excretion during the period a II (see fig.) in relation to the excretion during the period b II and a III—b III as well as a I—a II, a II—a III, b I—b II and b II—b III.) The results are all the more convincing as the calculations are made on the whole extent of each period. Thus they also comprise the determinations, which are still influenced by the diet conditions during the preceding period.

Thus the result implies that a certain increase in the excretion of ascorbic acid can be obtained by administering only riboflavine to a basic diet, which moreover contains aneurin and niacin, but that considerably higher values can be attained if the basic diet contains casein. Additions of aneurin, niacin, adermin or pantothenic acid do not by themselves affect the ascorbic acid excretion. The further increase, which is obtained by the addition of yeast, may be explained by the thereby increased provision of riboflavine, but can, of course, also depend on some other factor in the yeast.

In order to illustrate further the significance of the riboflavine, investigations have been carried out on the *ascorbic acid content in the liver* of rats with varying administrations of riboflavine. Two groups of rats, each comprising eight animals, were placed on the above-mentioned basic diet and in addition received casein up to 23 %, 3 mg. aneurin and 200 mg. niacin per 1000 g. diet, upon which a corresponding decrease was made in the wheat starch. The only difference between the groups as regards diet was that the one received an addition of circa 40 gamma riboflavine per day and per rat. After 24 days the content of ascorbic acid in the liver was analysed — titration in metaphosphoric acid with Tillman's reagent — and, as appears in *Table 1*, the liver in the animals which have received the riboflavine addition contains considerably greater quantities of ascorbic acid. The difference is statistically established. The Table at the same time shows liver weights and the liver weights' relation to the weight of the body in order to demonstrate that the difference in the ascorbic acid

Table 1.

Diet	Number of animals	Animals'		Liver weight	Liver weight body weight	mg % ascorbic acid in the liver
		initial weight	final weight			
Basic diet + B <sub>1</sub> + niacin + casein....	8	38.8	46.8	2.82	0.060	14.1 ± 0.66
Basic diet + B <sub>1</sub> + niacin + B <sub>2</sub> + casein	8	38.0	56	3.22	0.057	19.6 ± 1.12

content cannot have depended on changes in the relative size of the liver with different diets.

The addition of casein to the diet is necessary according to the excretion experiments for obtaining a maximal increase in the ascorbic acid excretion on the administration of riboflavine. The casein effect could either depend on the fact that casein is somehow or other necessary, for instance as raw material for the ascorbic acid synthesis, or on a stimulation of the ascorbic acid synthesis depending on the casein's content of cyclic amino acids in the same manner as when azotoluene, for example, is administered. With a view to investigating which is the essential mechanism, the following experiments have been carried out.

Two groups of rats, each comprising six animals were placed on the basic diet, to which were added 3 mg. aneurin and 200 mg. niacin per kg. of diet. To each animal 40 gamma riboflavine was given with the food in a cup daily. One group received, in addition, casein up to 23 % with a reduction of the starch as above. As is seen in *Figure 3* no difference appears in the ascorbic acid excretion in both groups. After 14 days both groups received azotoluene (0.3 % of the diet), upon which the ascorbic acid excretion rose from circa 0.4 mg. per 24 hours to 1.5—2 mg. in the group, which had received the addition of casein, whereas, on the other hand, the casein-free group showed an increase to 0.5—1 mg. After some time, however, casein was also given to the other group, and it is then found that the ascorbic acid excretion becomes the same in both groups. Thus the addition of casein as such has not affected the ascorbic acid excretion, but the increase, experienced on the administration of azotoluene, becomes considerably less if casein be lacking in the diet.

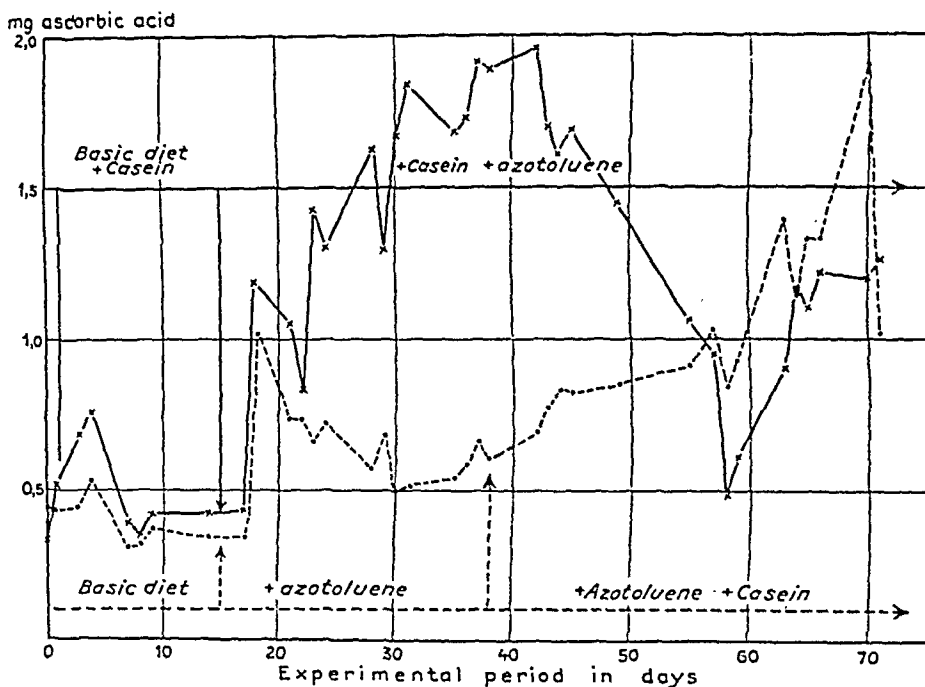


Fig. 3. Output of ascorbic acid in the urine of rats on a diet with sufficient addition of riboflavine and varying additions of casein and 2,3-azotoluene. Each curve corresponds to the average values for 6 animals.

## II. The variations noticed in ascorbic acid excretion and in the liver's ascorbic acid content depends on changes in the production of ascorbic acid.

Variations in the ascorbic acid content in the liver and ascorbic acid excretion in the urine could either depend on a synthesis of greater or lesser quantities of ascorbic acid, or on an influence on the excretion mechanism. With a view to ascertaining the cause of the changes observed, the following experiments were carried out.

Two groups of guinea-pigs, each consisting of six animals with a weight of about 250 g., were placed on a complete diet (HAMMARSTEN'S no. 211) and received daily by injection a constant quantity of ascorbic acid. To one or the other group alternately 200 gamma riboflavine was given daily, *i. e.* considerably more than the dose which in previous experiments had shown that it had influenced ascorbic acid excretion and ascorbic acid content in the liver of rats. This addition of riboflavine, however, did not result in any changes in the excretion of ascorbic acid. The guinea-pigs themselves are not able to synthesize any ascorbic acid. On the supply being constant, therefore, the excreted quantity should

furnish information about that which is metabolized in the body. Thus, as the excretion conditions in guinea-pigs are not influenced by the change in diet, it seems reasonable to suppose that the changes observed in the experiments with rats described above under I. have been due to variations in the synthesis of ascorbic acid and not to a changed metabolism of the latter.

III. *The effect of the secondary deficit of the ascorbic acid in rats on the symptoms of riboflavine deficiency in the diet.*

Thus it should have been clearly demonstrated that variations in the synthesis of ascorbic acid in rats can be brought about by variations in the administration of riboflavine. In order to make clear whether this secondary ascorbic acid deficit has any importance in regard to the symptoms of riboflavine deficit, experiments have been carried out partly on the period of survival and partly on detoxication reactions in rats; which do not receive sufficient riboflavine in the diet but instead get ascorbic acid.

Two groups of rats, the one comprising 14 experimental animals ("the control group"), and the other ("the experimental group") comprising 13 animals, received both the B-vitamin-free basic diet with the addition of 3 mg. aneurin and 200 mg. niacin per kg. diet and casein up to 23 %. The experimental group, in addition, received three times a week 20 mg. ascorbic acid by subcutaneous injection. The curves of growth for both groups were identical with an initial increase and following that a decrease in the body weight. The average length of life was, however, for the control group  $40.3 \pm 1.78$  days, whereas the average length of life in the group, which received ascorbic acid, was  $47.8 \pm 1.63$ . The difference between the two groups is significant.

From various quarters (for literature see EKMAN 1944) there has been a tendency to ascribe to ascorbic acid a rôle in the organism's detoxication ability, and the longer survival period in the animals which received ascorbic acid can possibly be explained by the fact that their detoxication of toxic metabolic products is greater than that of the control group. In previous papers (EKMAN 1944 and 1947) the opinion has been advanced that the detoxicating ability of ascorbic acid consists of an oxidizing decomposition of toxic compounds. The theory was based on results from experiments with guinea-pigs.

EKMAN-STRÖMBECK (1947) showed that rats, which receive a

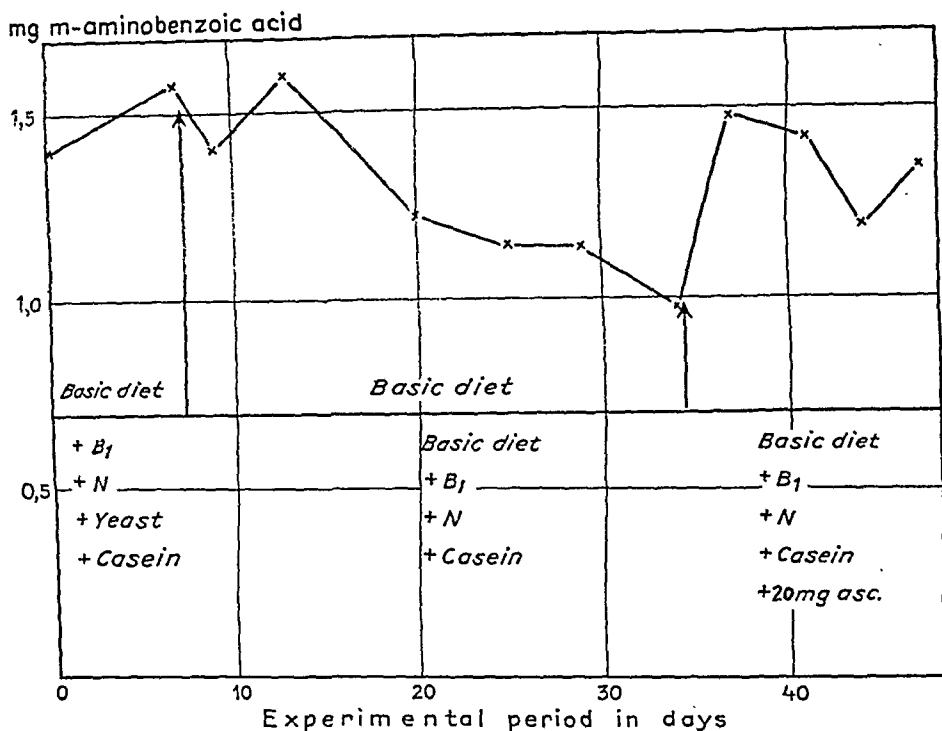


Fig. 4. Output of m-aminobenzoic acid in the urine of rats which daily received 10 mg. m-toluidine and various additions of yeast casein and ascorbic acid. The curve corresponds to the average values for 6 animals.

normal diet with 40—50 mg. sulfanilamide added, excrete roughly 50 % of the sulfanilamide quantity administered. If the animals receive the rice diet deficient in riboflavine and casein the sulfanilamide excretion is raised to roughly 100 %. This change in the excretory conditions came about slowly but was finished after 60 to 70 days. At the same time it was shown that running parallel with the increase of sulfanilamide excretion there was a decrease in the excretion of ascorbic acid. If the rats, which excreted roughly 100 % of the quantities of sulfanilamide administered, received daily 20 mg. ascorbic acid by injection, the excretion decreased again to the normal diet values of about 50 %.

The experiments were now built up in such a way that we examined the excretion in the urine of an oxidation product of m-toluidine, namely, m-aminobenzoic acid, 10 mg. m-toluidine was daily administered by pipette in the pharynx. Fig. 4 shows that when the rats received the previously mentioned basic diet with m-toluidine together with the addition of yeast and casein the 24-hour quantities of excreted m-aminobenzoic acid amounted roughly to 1.5 mg. With the yeast withdrawn from the diet the

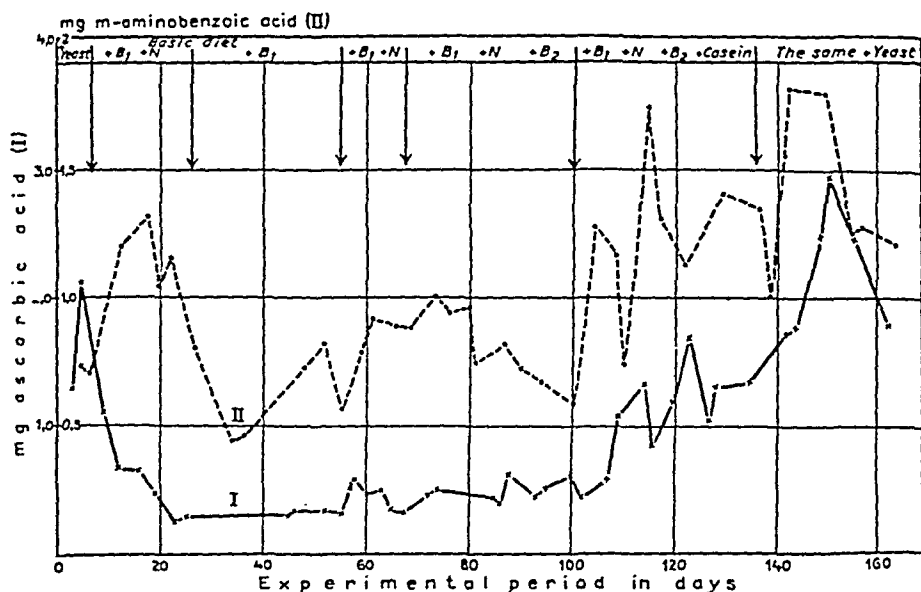


Fig. 5. Outputs of ascorbic acid (I) and m-aminobenzoic acid (II) in the urine of rats which daily received 30 mg. 2,3-azotoluene by mouth and varying additions of vitamins and casein to the basic diet. Each curve corresponds to the average values for 6 animals.

excretion decreased to roughly 1 mg. If the animals now received ascorbic acid the excretion again rose to roughly the original values. In another experiment we simultaneously studied the excretion of ascorbic acid and the excretion of m-aminobenzoic acid in varying additions to the basic diet. *Fig. 5* shows that a complete parallelism is obtained between both curves. With the addition of riboflavine and casein the excretion of both ascorbic acid and m-aminobenzoic acid increases; with the withdrawal of these components from the diet it decreases again.

### Discussion.

The experiments described above seem to show that additions of niacin, aneurin, adermin or pantothenic acid to a basic diet, which does not contain B-vitamin or protein, do not result in any changes in the excretion of ascorbic acid. This holds good whether the animals receive an addition of any aromatic compound or not. The addition of casein alone does not influence the ascorbic-acid excretion. The addition of riboflavine alone gives a certain increase of ascorbic acid excretion. A sharply increased excretion of ascorbic-acid is obtained with a simultaneous administration of riboflavine and casein. The ascorbic-acid content



in the liver was higher in rats, which had received both riboflavine and casein than in animals which had only received casein.

In various quarters it has been maintained that the initial material for the ascorbic acid synthesis in the rat organism consisted of carbohydrates. Thus GUHA and GHOSH (1935) could show that the liver tissue *in vitro* can form ascorbic acid from mannose. HOPKINS et al. (1936) found that only with a sufficient supply of carbohydrates were more important quantities of ascorbic acid found in the liver. Riboflavine, owing to its incorporation in certain coenzymes, assumes an important position in the intermediary metabolism in the organism. Possibly its importance for the ascorbic-acid synthesis may be set in connection with an effect on the carbohydrate metabolism.

Our experiments seem to indicate that casein is necessary for the ascorbic-acid synthesis. Whether it plays any rôle as initial material or in some other way cannot be determined from our experiments.

The investigations further show that the changes observed in the excretion are dependent on variations in the synthesis of ascorbic acid and not only variations in the latter's metabolism. A rat, receiving a diet deficient in riboflavine and casein, seems, therefore, to at least to a certain degree dependent on the supply of ascorbic acid from outside and thus comparable with, for instance, a guinea-pig. By experiments on survival we have been able to demonstrate that such rats live longer when they receive ascorbic acid, and furthermore that the supply of ascorbic acid normalizes the oxidative detoxication of aromatic compounds in the organism. The opinion put forward in previous papers (EKMAN 1944 and 1947) that the function of ascorbic acid in the normal organism consisted, among other things, of a detoxication of toxic compounds through oxidizing processes would seem on this account to obtain substantial support.

The present investigations are due to observations made in connection with investigations on the metabolism in carcinogenic azocompounds. In various papers (KENSLEY et al. 1941) it has been shown that the appearance of liver tumours after administration of dimethylaminoazobenzene has been restricted by administration of riboflavine and casein. STRÖMBECK (1946), in previous investigations, found bladder tumours after administration of azotoluene, when the animals received a diet mainly consisting of rice flour, whereas, on the other hand, no tumours appeared

when the animals received HAMMARSTEN's (1937) diet no. 211. Among the differences between both diets especial interest attaches to the low content of riboflavine and protein in the rice diet. The results set out now and in earlier papers (EKMAN and STRÖMBECK 1947) seem to indicate that the mechanism in the combination riboflavine-casein as tumour-protective factors may be due to the dependence of ascorbic acid synthesis on a sufficient supply of riboflavine and protein. On studying the azotoluene metabolism it was demonstrated that relatively large quantities of the oxidized decomposition products of azotoluene (aminobenzoic acid and aminocresol) were excreted on the tumour-protective diet, whereas, on the other hand, no or only inconsiderable quantities of toluidine were excreted. On the tumorigenic diet, however, small quantities of oxidation products and increased quantities of toluidine were excreted. Our investigations seem to be in favour of the variations described in the metabolism being secondary to the variations in the ascorbic acid synthesis. Only when this takes a normal course, does a normal detoxication through oxidizing processes occur.

In other investigations also the possible significance of ascorbic acid synthesis in connection with the appearance of tumours has been observed. KENNAWAY et al. (1944 b) were able to show that with the injection of certain carcinogenic substances the content of ascorbic acid in the liver of mice was increased. The results could be compared with observations that the content of ascorbic acid in the liver showed itself to be higher in a mice stock with a high spontaneous cancer frequency than in a mice stock with a low frequency (KENNAWAY et al. 1944 a). Later KENNAWAY and BAFF (1946) report that deficit of aneurin or riboflavine in the diet resulted in a decreased content of ascorbic acid in the liver of mice.

As previously mentioned vitamin A has also been placed in connection with the ascorbic acid synthesis in different animals. It may, therefore, be of interest to mention that, on administering oestrogenic substances to rats, which received a diet deficient in A-vitamin, bladder tumours were obtained (HUME et al. 1939). It is possible that the importance of the low A-vitamin content in the diet has depended on the reduced ascorbic acid production. It has also been maintained by KENNAWAY and TIPLER (1947) that a detoxication of sterin-derivatives normally occurs through ascorbic acid during gravidity.

Thus both A-vitamin and the combination casein-riboflavine can restrict the appearance of tumours during certain experimental conditions in rats. Against the background of the investigations put forward here it seems remarkable that both seem to be necessary for the ascorbic-acid synthesis in rats. Investigations into a possible effect through vitamin A on the metabolism of a carcinogen, *e. g.* azotoluene, are planned.

### Summary.

I. The ascorbic acid excretion in the urine has been studied in rats on varying diets. A normal excretion could be maintained, when the diet contained sufficient quantities of protein (casein) and riboflavine. Addition of aneurin, niacin, adermin and pantothenic acid had no effect. This also held good when the ascorbic-acid excretion was increased by the administration of an aromatic compound, *e. g.* azotoluene. The experiences from these experiments indicate that the casein is necessary for the synthesis of ascorbic acid and does not merely have a stimulating effect, as *e. g.* azotoluene; an increased ascorbic-acid excretion after administration of azotoluene was only obtained when the diet contained sufficient quantities of casein (and riboflavine).

In agreement with the excretion experiments rats, with a deficit of riboflavine show a decreased ascorbic-acid content in the liver.

II. The variations observed in ascorbic-acid content in the liver and in the ascorbic-acid excretion should correspond to the variations in the synthesis of ascorbic acid and not only to a varying metabolism.

III. If rats with a deficit of riboflavine receive ascorbic acid, the period of survival is increased.

In experiments with administration of sulfanilamide to rats ca. 100 % of the quantities administered were excreted at a riboflavine deficit; after administration of riboflavine *or* ascorbic acid only about 50 %. In experiments with administration of azotoluene only small quantities of the oxidized decomposition product m-aminobenzoic acid were excreted in riboflavine deficit; after supply of riboflavine (in a sufficient addition of casein) *or* ascorbic acid, considerably increased quantities were excreted.

It is considered that the ascorbic acid is of importance for oxidizing detoxication processes in the organism of the rat. Ribo-

flavine, by its effect on the ascorbic-acid production, can have a similar influence.

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## The Effect of Illumination upon the Sensitivity of Isolated Retinal Elements to Polarization.

By

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In the first observations on the effect of polarizing currents on the discharge of isolated elements in the cat's retina (GRANIT, 1946) it was noted that just as polarization influenced the threshold to light so also did illumination change the threshold necessary to elicit a discharge by polarization. Neither effect was seen with all elements. The influence of polarization upon stimulation with light has since been analyzed from various points of view by GERNANDT (1947) and GRANIT (1948 a, b). This paper will briefly describe some results obtained in the reversed experiment when the threshold of polarization was measured both in the dark and *during* illumination with light of known multiples of the threshold effect.

Four questions have been raised in the present experiments. (i) Can illumination depress the electrical threshold? (ii) Can illumination facilitate the electrical threshold? (iii) Can anodal elements become cathodal or, *vice versa*, cathodal ones anodal under the influence of illumination? (iv) Do the electrical and light thresholds after illumination return to normal along similar curves?

### Method.

The fully dark-adapted eye of the decerebrate cat was used together with the micro-electrode technique for isolation of retinal elements in the manner developed in this laboratory and several times described

(e. g. GRANIT, 1947). Polarization electrodes were inserted in the nasal and temporal corners of the eye. The anodal elements then respond to the make of the anodal current, the cathodal ones to the make of the cathodal current. The micro-electrode picks up the discharge on the retina just inside the nasal electrode which determines the polarity. Current reversal produces off-discharges in both types of elements at cessation of polarization, in both cases also preceded by inhibition. Similarly the on-effects to the make of the current, for both types of element, are succeeded by inhibition at the break (see particularly GRANIT, 1948 a).

The experiments were begun by determining the polarity of the element isolated, i. e. by finding out whether the on-effect was elicited by the anode or the cathode, as stated above. Then the thresholds in milliamperes for both anodal and cathodal effects were measured for 3 sec. of polarization. This must not be done too rapidly or the elements are liable to vary in sensitivity to the current. Sometimes the thresholds varied too much for quantitative work but generally they did not do so and could be used in the experimental analysis. The next step consisted in measurements of the light thresholds at "on" and "off" for 3 sec. exposures to some wave-length from our Wright colorimeter (WRIGHT, 1946), mostly  $0.510 \mu$ , but other wave-lengths were also tried. The off/on-ratio at the threshold varied from element to element as pointed out before (GRANIT and TANSLEY, 1948). When afterwards the eye was illuminated with a stimulus 1,000 times the threshold-light this factor was always based on the more sensitive of the two components. Occasionally stronger non-spectral stimuli were used, generally the Ilford spectral filters for red, green and violet, placed in a beam of 800 m. c.

The polarization device, with its commutator for changing the direction of the current and a high resistance in series with the eye, was switched on and off by hand and so it was impossible to measure the polarization thresholds *during* illumination very quickly. However, when large changes were obtained they were generally noted already within the first minutes of illumination. The element refused to respond to the polarizing current that had elicited a discharge in the dark-adapted eye.

The eye was kept illuminated for 10—20 min. during which final values were obtained for the change in the electrical threshold. Then followed recovery in the dark and measurements of the light and polarization thresholds until both had become normal. If the thresholds did not recover the results were considered unreliable even though, to all appearance, the spike was as perfect as in the beginning.

## Results.

We can describe the results very briefly because their interpretation is so difficult that very little is gained by giving more details than are necessary for answering the questions raised

above. In all, successful measurements were carried out with 28 elements.

(i) Illumination at 1,000 times threshold strength can raise the threshold to polarization in all types of element but does not necessarily do so in all elements. Some remain uninfluenced but in many of the uninfluenced elements a definite decrease of electrical sensitivity was noticed when stronger lights were used (8—800 m. c. or Ilford filters in the 800 m. c. light beam). Others withstand strong illumination without change of electrical threshold. The amount of change varies so much from element to element that it proved impossible to unravel any correlations with respect to type of element or off/on-ratio. The typical effect of illumination was a depression of the electrical threshold which generally was instantaneous but sometimes the threshold decreased or increased during continued illumination. In this material the anodal elements proved to be more sensitive to the effect of illumination on the polarization threshold than the cathodal ones.

Some examples will show the order of magnitude of the maximal changes when both the on- and the off-component were affected symmetrically. The letters D and L will be used to denote the dark- and light-adapted states respectively. The values are given in milliamperes. 0.05D—0.1L, 0.075D—0.50L, 0.3D—0.7L. These three elements were anodal and illuminated at 1,000 times the threshold. There were four pure on-elements but in all of them the effect was small or absent though in one definite. The on-elements, as pointed out before, are cathodal (GERNANDT and GRANIT, 1947). The effects were generally small or absent in the cathodal on/off-elements too but it proved possible in most of them, by increasing the strength of illumination, to obtain definite and easily measurable drops in the electrical thresholds. The following are examples of such cathodal elements for which stimulus strength had been increased by using the Ilford spectral green filter: 0.075D—0.35L, 0.2D—0.35L, 0.25D—0.43L. The transmission of the 800 m. c. stimulus is reduced to about 10 % by the Ilford green filter.

(ii) Can facilitation of the electrical threshold be demonstrated? Whereas the polarization thresholds several times proved to be uninfluenced by illumination it was difficult to be quite certain about the existence of facilitations. It is true that there were some cases in which facilitation was noted but the effects were

small. Perhaps the safest statement at the moment is that facilitation seems probable (see below).

(iii) Do shifts of polarity occur? Such shifts are sometimes regularly obtained, indeed, a modest degree of asymmetry of the effect of illumination on the opposite poles needed for make- and break-discharges is quite common. Greater degrees of asymmetry amounting to complete shifts of polarity have also been seen. Since these cases are very interesting some examples will be given.

An anodal element with an off/on-ratio of 1.8 at wave-length  $0.510\mu$  gave the characteristic anodal make- and cathodal break-discharge to polarization between 0.20—0.25 mA. Immediately after illumination with the test light (1,000 times the threshold) the element refused to respond to polarization with 0.5 mA. Somewhat later, during polarization, it showed reversed polarity and responded with a *cathodal* make- and break-discharge to 0.38 mA and occasionally with an anodal break-discharge to 0.25 mA. There was full recovery in the dark and the electrical threshold also returned to normal. The element again became anodal. Repetition of the illumination experiment, this time performed at wave-length  $0.460\mu$  at 1,000 times threshold strength. Complete reversal of polarity with cathodal make- and anodal break-discharge already for 0.15 mA after 4 min. of illumination. Afterwards complete recovery of light threshold and the polarity swung back to normal together with the return of the original polarization threshold. The element again became anodal and required 0.25 mA to respond. This is one of the cases where also a real facilitation of the polarization threshold seemed probable.

A cathodal element with off/on-ratio 16. Before illumination the polarization threshold was 0.2 mA. At this strength the element was giving a good cathodal make- and an anodal break-discharge. Illumination with Ilford spectral violet filter in the 800 m. c. light beam. The polarization threshold fell to 0.5 mA; there was now anodal make- and cathodal break-discharge. Thus complete reversal of polarity. Full recovery afterwards in the dark. After 5 min. the element again became cathodal and responded to 0.25 mA with strong typical effects.

A third element that reversed its polarity did not return to normal after illumination and maintained its shift of polarity. The off/on-ratio also changed from 0.042 to 0.11 but since this



took place at a higher absolute level of sensitivity to light it was impossible to conclude that the element had been damaged in the course of the experiment. The spike was perfect all the time. There were no adjustments necessary to maintain the spike in the course of the experiment and a very large number of readings were taken. It was several times illuminated and always made a rapid recovery.

Since in addition several elements underwent minor shifts of polarity we conclude that illumination can re-balance certain elements suggesting very definitely that the on/off-element consist of anodal and cathodal components either of which may be suppressed or, perhaps, enhanced by illumination. To be noted is that all these effects are obtained with illuminations of relatively modest strength. With strong lights we have sometimes seen very large depressions of the electrical threshold.

(iv) The relative rates of recovery of light and polarization thresholds may vary from element to element but the general rule noted was that the polarization thresholds recovered very quickly and long before the light thresholds, particularly in cases where the latter underwent large depressions leading to delayed recovery (GERNANDT, 1948).

Finally remains to be mentioned that the changes of the polarization thresholds did not necessarily require changes in the spontaneous frequency of the discharge. The spontaneous rhythm did, of course, change very often as a consequence of illumination but in some cases this effect was modest or absent, in others the rhythm re-established itself later during illumination without concomitant changes in the polarization thresholds. The change in the latter were not therefore secondary to the variations in the spontaneous frequency.

### Discussion.

Since light elicits large changes of potential in the retina it seems natural to compare the effects of illumination upon the polarization thresholds with the effects of polarization on the electrical thresholds of nerves. These are known to be considerable. It is well known from psychophysical work that the state of adaptation influences the electrical thresholds (see e. g. SCHAEFER, 1942) and the essential question raised by this work

is therefore whether the analysis of single elements can add anything to our knowledge of such phenomena.

In this respect there is an interesting parallel with the reversed type of experiment dealing with the effect of polarization upon the light threshold. In these experiments too (GRANIT, 1948) the light thresholds of the anodal elements were highly sensitive to polarization, those of the (always) cathodal pure on-elements practically not at all, and those of the cathodal on/off-elements were generally difficult to influence, but a minority of the cathodal elements was as sensitive as some anodal ones. In the anodal ones it was particularly easy to elicit strong inhibition of the light threshold by polarization with the depressing (cathodal) pole.

All these results were interpreted to signify that the simple cathodal on-elements were deficient in structures sensitive to polarization, the anodal elements amply supplied with such structures, the majority of the cathodal on/off-elements being more like the pure on-elements whilst the minority behaved like the anodal ones and possessed these structures.

From this point of view it seems of fundamental importance that only a certain number of the elements, and particularly some anodal ones, are so designed that the modest light adaptation used in these experiments easily raises the electrical threshold. This result suggests that the direct path through receptor, bipolar and ganglion cell, shared by all elements, is deficient in structures which are capable of being influenced by modest light adaptation so as to make the element relatively more insensitive to electrical stimulation.

Clearly the direct path through receptor, bipolar and ganglion cell is neither very sensitive to polarization (GRANIT, 1948 a) when tested with the light threshold nor is its polarization threshold particularly sensitive to the changes set up by illumination. The former experiment was easier to perform and so it could be shown that the curves relating light thresholds to polarization strength in the case of the anodal elements differed fundamentally from those of the pure on-elements which could be regarded as prototypes of pure cathodal elements (GRANIT, 1948 a).

In both types of analysis the anodal on/off- and pure off-elements appear to be extremes representing one type of design whereas the majority of the cathodal on/off-elements tend to approach the cathodal pure on-elements as the other extreme.

The latter seem to be deficient with respect to some structures possessed in abundance by most anodal elements. The earlier experiments on the effects of polarization upon the light thresholds suggested that these structures must be internuncials. Accordingly these internuncial neurones of the retina appear as structures which, when polarized, easily set up potential changes influencing the light thresholds or *vice versa*, when illuminated, easily set up potential changes with the result that the electrical threshold is raised. Light and polarization are to some extent interchangeable with respect to the internuncials with which the anodal elements are particularly well supplied.

These results are thus interpreted to mean that certain internuncial cells represent an organ designed for the production of electrical potential serving the ultimate purpose of directing and controlling the internal switchboard and thereby also the off/on-ratio. This mechanism was used by one of us (GRANIT, 1948 b) to study the colour sensitivity of the retinal elements.

The fact, that the polarization thresholds returned to normal after light adaptation before the light thresholds did so, shows that what might be called "photochemical sensitivity" is something very different from what is being measured by the polarization threshold. Hence the depression of photochemical sensitivity may persist after the time when the electrical test shows that the "internal switchboard" is functioning normally.

The cases in which a shift of polarity were noted seem to us valuable as a warning against too much schematization. They show that some elements contain both anodal and cathodal components and that definition by polarity thus to some extent depends upon what components happen to be in the majority. There is apparently a zone of overlap of cathodal and anodal properties between the theoretically pure anodal and cathodal elements. Whether such shifts of polarity are due to blocking potentials or to active withdrawal of certain synaptic knobs under the influence of electrical forces we do not know. The second possibility should not be left out of sight.

Long ago one of us (GRANIT, 1938) introduced the concept "electro-adaptation" to account for the fact that the light-adapted frog's retina had a more prominent negative component than the dark-adapted one and that certain other potential changes characterized light adaptation. The idea that potential changes can re-balance an organ such as the retina is therefore not based

on the present results alone. (For further discussion, see *e. g.* GRANIT, 1947.)

### Summary.

Polarizing electrodes were placed in the nasal and temporal corners of the eye of the dark-adapted decerebrate cat and the threshold discharge of retinal elements to polarization and illumination was measured by studying the spikes isolated by a micro-electrode in the eye.

In certain types of element the threshold to polarization rose when the eye was illuminated at 1,000 times threshold strength, in others not.

Some elements that in the dark had a lower threshold for anodal currents became more sensitive to the cathode during illumination and, *vice versa*, some elements with lower thresholds to cathodal stimulation became more sensitive to anodal stimulation during illumination.

After illumination the polarization thresholds often returned to normal before the thresholds to illumination did so.

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## Further Observations on Environmental Factors Influencing the Temperature Sensitivity of Mammalian Nerve Fibres. Effects of $\text{Ca}^{++}$ and Other Agents.

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In earlier work it has been shown (LUNDBERG 1948) that information about different types of fibres may be gained by examining their temperature sensitivity. The membrane potential and negative afterpotential were maximal at about  $37^\circ$  in A fibres (motor root), in C fibres, however, at  $25^\circ$ . Also the spike potential of these fibres showed different sensitivity to temperature, the spike of the motor alpha fibres was maximal at  $30\text{--}35^\circ$  and conduction was blocked at about  $10^\circ$ , whereas the C spike was maximal at a much lower temperature and conduction was not blocked until cooling went below zero degrees. These differences became of particular interest when, in the same work, it was shown that the temperature sensitivity of the nerve fibres were functions of the concentration of potassium ions in the external medium. If the potassium concentration is increased the membrane potential and the spike potential reach their maximum value at a higher temperature than normally, and, if the nerve is treated with potassium-free solution, the membrane potential, spike potential and negative afterpotential have their maxima at a lower temperature than normally. Continued analysis of how ionic factors influence the temperature sensitivity of nerve fibres may therefore be of importance for understanding the processes determining the differential sensitivity to temperature changes.

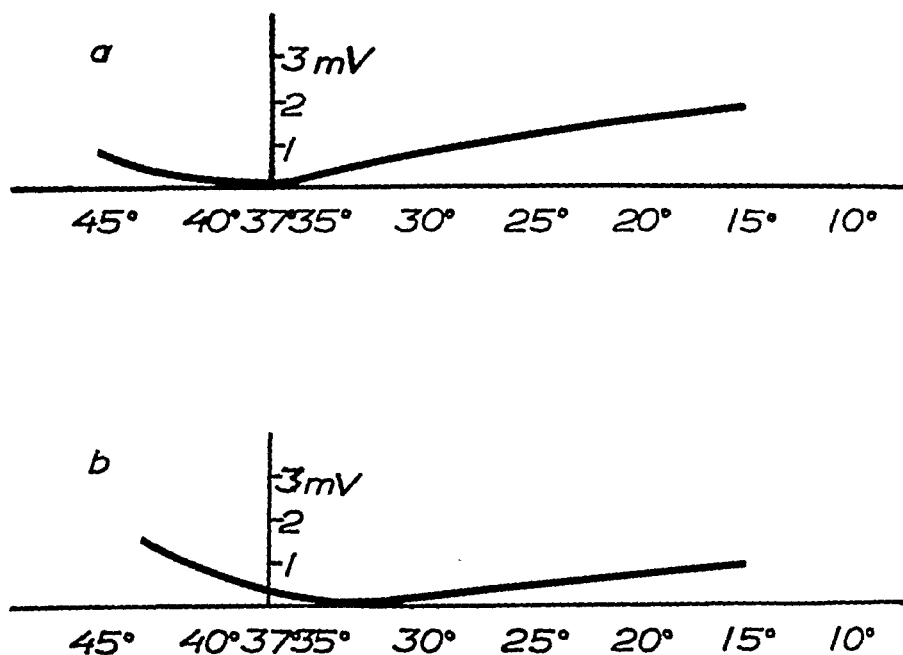


Fig. 1. Membrane potential in motor S1 root as function of temperature. a. Normal root. b. On the same root after treatment with Krebs' solution containing 150%  $\text{CaCl}_2$  above normal. The temperature range for minimum ordinates represent the maximum of membrane potential.

This paper deals with the effect of the variation in the amount of  $\text{CaCl}_2$  in the external medium of the nerve fibres. The action of magnesium ions, phosphate ions, asphyxia and ether have also been studied. To the best of our knowledge very little has been reported in the literature about the effect of ionic factors on the temperature sensitivity of nerve fibres. ETS and BOYD (1938) who discovered that excess potassium caused the cold block to shift to a higher temperature range found also that  $\text{CaCl}_2$  did not counteract this effect of potassium ions.

*Method:* L7 and S1 motor roots from cats were used. The roots were excised, kept in Krebs' solution and aerated with 93.7 %  $\text{O}_2$  and 6.7 %  $\text{CO}_2$ . The nerve was subjected to local temperature changes with the aid of a one cm long, lacquered silver thermode containing a groove for the nerve. As to technique of recording see LUNDBERG 1948.

## Results.

The block of conduction that shifts to a higher temperature after treatment with excess potassium can be shifted back again if in addition the amount of  $\text{CaCl}_2$  is increased. The effect when only

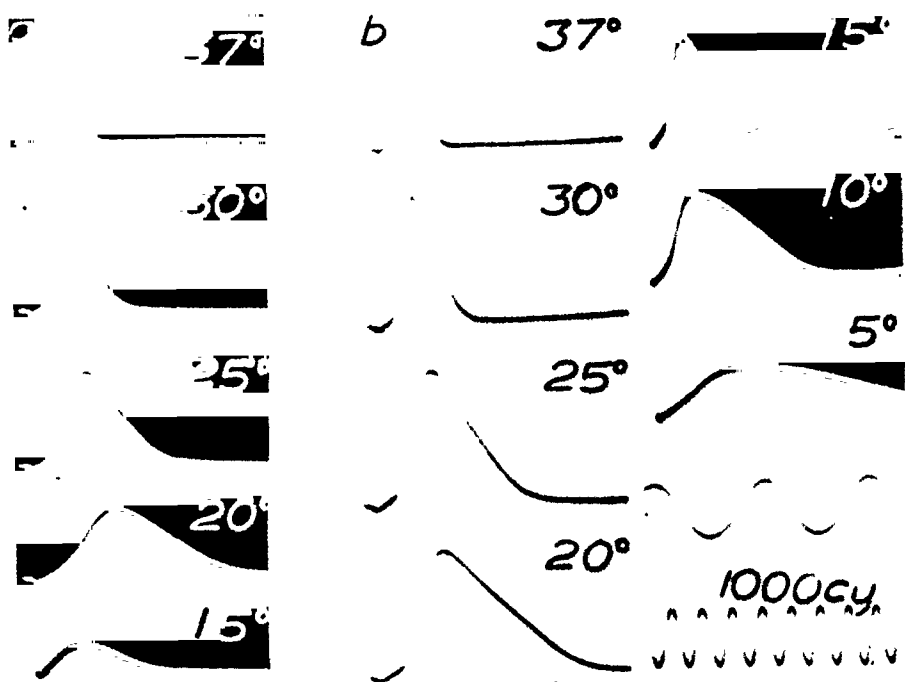


Fig. 2. Motor S1 root, cat. Maximal stimulation of alpha fibres. Root which had been kept in normal Krebs. The same root after 15 minutes treatment with Krebs' solution containing 150 %  $\text{CaCl}_2$  above normal.

$\text{CaCl}_2$  is increased corresponds to the one obtained when the concentration of potassium ions is lowered. Fig. 1 demonstrates that after treatment with excess  $\text{CaCl}_2$  (fig. 1 b) the membrane potential has its maximum at a lower temperature than normally (fig. 1 a) and the drop of potential to warming is larger, to cooling, however, smaller than in the normal root (a). Fig. 2 shows how the normal temperature sensitivity of the spike potential (fig. 2 a) is changed after treatment with excess  $\text{CaCl}_2$  (2b). The effect is qualitatively the same as that observed after treatment with potassium-free Krebs *i. e.* a lowering of the temperature for maximum, spike height and an increased resistibility to cold. Fig. 3 shows the temperature effect on the negative afterpotentials in normal root (a) and after treatment with a solution containing  $\text{CaCl}_2$  150 % above normal (b). Contrary to what is known for frog nerves (H. T. GRAHAM, 1933) there is a shortening of the duration of the negative afterpotential in excess  $\text{CaCl}_2$ . The detailed analysis of the negative afterpotential of mammalian nerve fibres is a difficult task, as has been shown by LORENTE DE NÓ (1947) but it is evident from fig. 3 a and b that the temperature

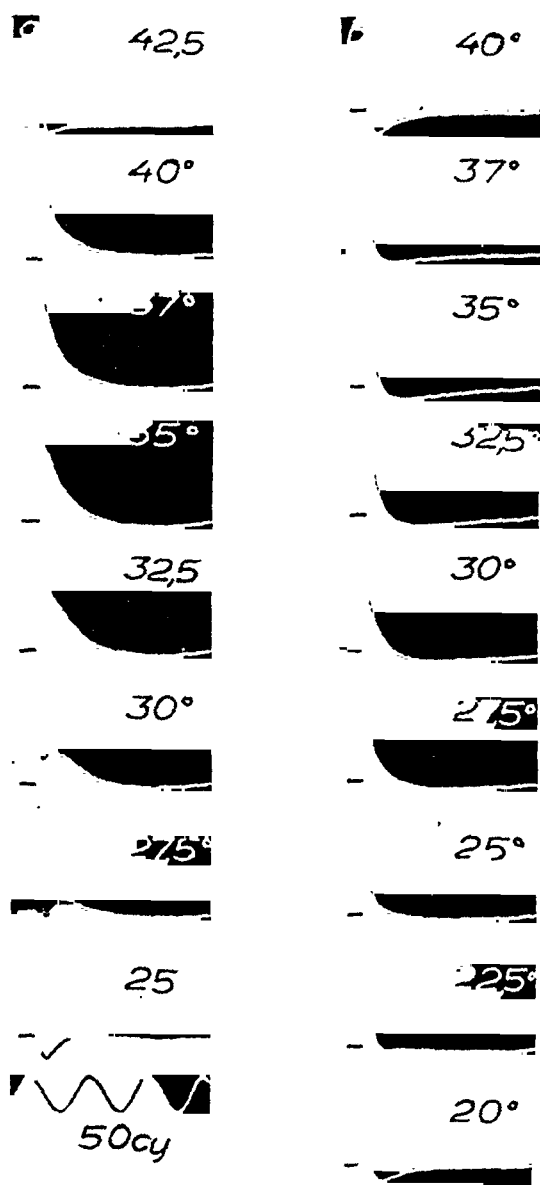


Fig. 3. S1 motor root, cat. Maximal stimulation of alpha fibres. Afterpotential recorded at different temperatures. a. Normal root. b. Root after 15 minutes treatment with Krebs' solution containing  $\text{CaCl}_2$  150 % above normal.

sensitivity of the negative afterpotential is altered in the direction of an increased sensitivity to high temperature and a greater resistance to low temperatures. This makes it reasonable to assume that the changed temperature sensitivity of the membrane potential after treatment with excess  $\text{CaCl}_2$  mostly is due to a change in the behaviour of the L fraction of Lorente de Nó (1947). Restitution to normal after treatment with excess  $\text{CaCl}_2$  is slow if the increase has exceeded 100–150 % and may, in fact, require



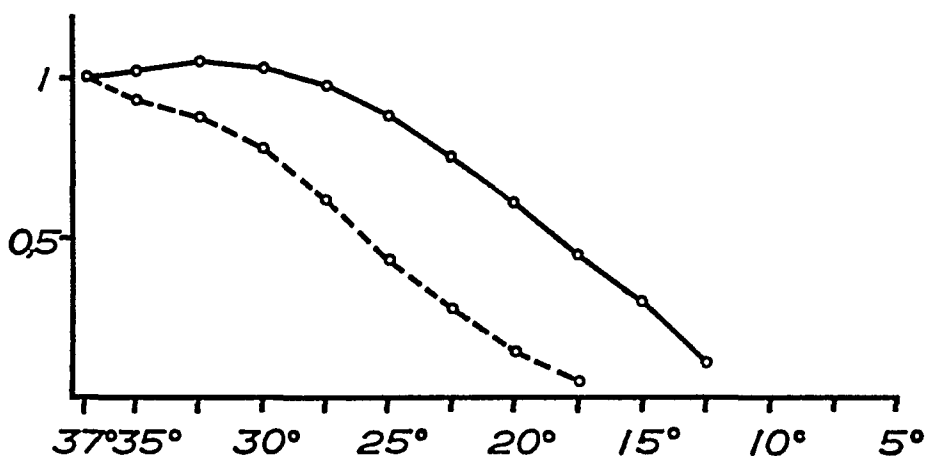


Fig. 4. S1 motor root, cat. Supramaximal stimulation of alpha fibres. Spike recorded at different temperatures in multiples of the height at 37° in each experiment. Line drawn in full normal root. Dotted line the same root after 150 min. treatment with Ca-free Krebs.

several hours. Using greater concentration of  $\text{CaCl}_2$  LORENTE DE NÓ (1947) found the nerve fibres to be irreversibly damaged. The slow restitution in our experiments is therefore probably due to some damage of the nerve fibres which had taken place despite of the fact that the concentrations used were relatively low.

We have also made experiments in order to see if Ca-free Krebs changes the temperature sensitivity of the motor roots. Only the temperature sensitivity of the spike potential was examined. The effect is demonstrated in fig. 4 and as will be seen the spike maximum shifted to a higher temperature together with the cold block. It must be emphasized that the effect is a slow one. It is visible first after two hours' treatment with Ca-free solution. Nevertheless the original sensitivity was restored. Only in a few of these experiments did we notice that the Ca-free solution caused spontaneous activity at 37°. A constant effect, however, was the sensitization of the discharge to cooling, an effect that was developed already after a few minutes and then did not increase much with time of treatment.

The effects on the temperature sensitivity of magnesium ions, phosphate ions,  $\text{CO}_2$ , ether and asphyxia were also examined.  $\text{MgCl}_2$  0.006 M (four times the normal amount in Krebs' solution) changes the temperature sensitivity of the spike potential in the same direction as does excess  $\text{CaCl}_2$ , phosphate ions 0.03 M ap-

plied as primary and secondary sodium phosphate mixed to give a pH of 7.3 made the spike maximal at about 37° and the cold block appeared at a temperature 5° higher than in untreated nerve. Lowering of the CO<sub>2</sub> tension below 1 % was equivalent with treatment with phosphate, whereas an increase to 14 % from the normal 6.3 % had no visible effect on the temperature sensitivity.

The experiments that were performed with ether and asphyxia are of interest in connection with results that previously have been obtained in experiments in which the concentration of potassium was varied (LUNDBERG 1948). When the potassium concentration of the external medium was increased the conduction block to cooling appeared at a higher temperature until finally the increase had become large enough to block conduction already at 37°. But if, at that moment, the potassium-treated segment was warmed above 37° conduction again became possible. It was also noted that on warming of the potassium-treated root there was a repolarization of the membrane leading to a shift of its maximum potential to 43° from the normal value around 37°.

Now the principal action of an increased concentration of potassium ions on nerve fibres is depolarization and therefore it is of interest to examine how other depolarizing agents influence the temperature sensitivity of the nerve fibres. It was found that asphyxia gradually shifted the cold block to a higher temperature until conduction ceased already at 37°—38°. If, at that moment, the root was warmed above this temperature it still would not conduct, nor did we in any phase of asphyxia find a repolarization of the membrane potential when warming from 37°. The effect of ether, locally applied to the part of the nerve within the thermode, was the same as that caused by asphyxia; the cold block moved up to progressively higher temperatures and when conduction finally was blocked at 37°—38° the fibres did not resume conducting when the nerve was warmed from that temperature. It has earlier been suggested (LUNDBERG 1948) that the effect of excess potassium on the temperature dependence of action- and membrane-potential is not due only to the depolarizing action of potassium ions. This is supported by the observation that ether and asphyxia do not have the property of moving the maxima of membrane potential and spike potential to a higher range of temperature.

### Summary.

This paper contains a description of the effect of  $\text{Ca}^{++}$  on the temperature sensitivity of membrane potential, negative afterpotential, and spike potential (motor A fibres, cat). The effect of  $\text{Mg}^{++}$ , phosphate ions, variation in the  $\text{CO}_2$ -tension, ether and asphyxia has been studied on the temperature sensitivity of the spike potential.

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## On the Effect of $K^+$ and $Ca^{++}$ on Thermal Stimulation and Spontaneous Activity of Mammalian Nerve Fibres.

By

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It is an old observation that rapid temperature changes can excite nerve fibres (see GRÜTZNER 1878). BERNHARD and GRANIT (1946) found that both local heating and cooling from  $37^\circ$  caused a discharge in mammalian nerve fibres. Simultaneously with the discharge there was a drop of potential relative to normal nerve in the region of the nerve in which the temperature had been changed. They assumed that the heat or cold potential thus created, served as "generator potential" initiating the discharge of impulses. C. v. EULER (1947) demonstrated that cooling selectively stimulated large myelinated fibres whereas heating stimulated afferent thin myelinated and unmyelinated fibres. EULER also studied the effect of temperature changes on the nerve membranes and found that local heating to  $45^\circ$  depolarized the thin fibres, local cooling the thick myelinated fibres.

In order to formulate the problem of these experiments we refer to fig. 1 (LUNDBERG 1948, fig. 5). As seen in the figure the membrane potential of the A fibres (motor root) is maximal at  $37^\circ$ , but that of the C fibres (splenic nerve) at about  $25^\circ$ , and, confirming EULER, it is also seen that warming to  $45^\circ$  gives a greater depolarization in C fibres than in A fibres, whereas to cooling the depolarization is mainly found in the A fibres. Now it was found (LUNDBERG 1948) that if the motor roots are treated with  $K^+$ -free

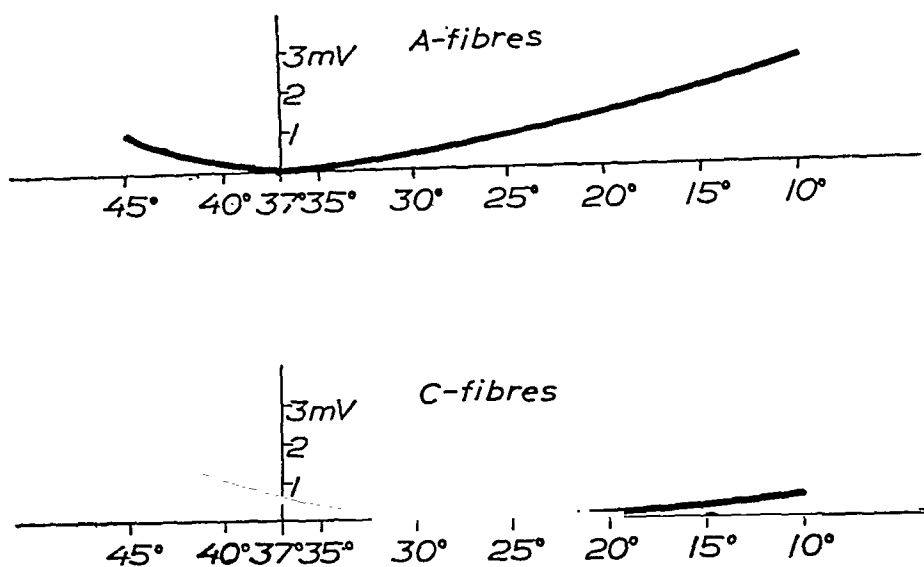


Fig. 1. The membranes potential of A and C fibres as functions of temperature. The temperature ranges for minimum ordinates represent the maximum of membrane potential.

Krebs solution, the A-curve of fig. 1 is shifted to the right so that it resembles the normal curve of the C fibres. Moreover, if the C fibres are treated with 2 or 3 times the normal  $K^+$ , their curve (Fig. 1) is shifted to the left so that it resembles that of normal A fibres. If then the excitation of A and C fibres by lowering or raising the temp. (resp.) were due to the thermopotentials, we should expect the effect to be reversed after suitable  $K^+$  treatment.

This is investigated in the first part of this paper.

### Methods.

Experiments with A fibres were made upon the excised motor roots from the cat, set up so that 1 cm lay within the thermode and the cut end dipped into a bath of Krebs solution. This was at temp. 37°, pH 7.3 and aerated with 93.7 %  $O_2$  6.3 %  $CO_2$ . The thermopotentials were recorded by a DC amplifier between AgCl electrodes, one within the thermode and one (earthed) in the bath. A condenser coupled amplifier served for the recording of massed discharges from the peripheral end of the root. The effects were observed on the screen and also led to a diode rectifier integrating the discharge.

Experiments on the heat-sensitive thin fibres were made on sensory roots cut peripherally. Rise of blood pressure, measured in the common carotid artery, was used as an indicator for excitation (C. v. EULER 1947). In these experiments the cats were decerebrated.

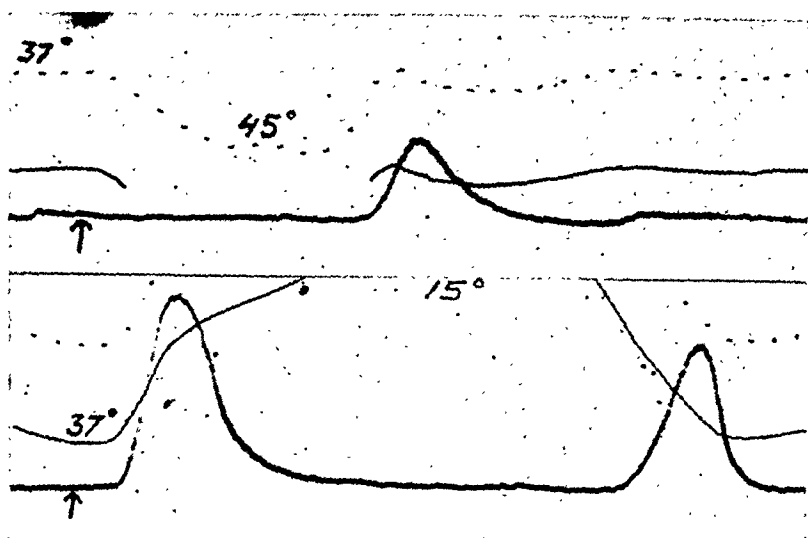


Fig. 2. Motor root, cat. Integrated discharge (arrows) at heating to 45° and at cooling from 37°. Time 1 sec.

## Results.

### I. Potassium and selectivity of thermal discharge.

In experiments made on motor roots it was found that cooling from 37° elicits a discharge, just as it does with nerves in situ. The discharge was seen in most of the examined roots but only during the first hours of the experiment. Fig. 2 demonstrates the integrated discharge. Even a few degrees of quick cooling caused an outburst of impulses and during continued cooling this discharge increased to become maximal at about 25° thereafter to decline rapidly. When the temperature again was raised to 37° another burst of impulses was seen. Heating to 45° never gave a discharge in motor roots. When the temperature from this range again was lowered, as in the upper record, this often activated the nerve (as described by C. v. EULER 1947).

Roots in which there was a discharge to cooling were selected for treatment with potassium-free solution. This treatment lasted for 5—30 minutes in various experiments. In practically every experiment the discharge to cooling persisted and appeared when the temperature was lowered a few degrees below 37°. Fig. 3 illustrates an experiment in which the integrated discharge and the change in the membrane potential of the nerve in the thermode were recorded simultaneously. Before the record was taken the nerve had been treated for 30 minutes with potassium-free Krebs

solution and afterwards it was found that the membrane potential was maximal at about  $30^\circ$  instead of at  $37^\circ$  as in fig. 1 illustrating the normal behaviour. In fig. 3 cooling from  $37^\circ$  to  $33^\circ$  caused a repolarization of the membrane and, nevertheless, simultaneously a discharge. In no case did we observe that heating to  $45^\circ$  elicited a discharge in a root treated with potassium-free Krebs.



Fig. 3. Motor S 1 cat. Simultaneous record of integrated discharge I and changes in the membrane potential P at cooling. Deflection downwards of P positivity and therefore there is repolarisation when the discharge is started. Time 1 sec.

For the interpretation of this experiment it is important to recall that the negative afterpotentials of the motor root alpha fibres on cooling diminish in normal roots, but increase in roots treated with potassium-free Krebs. These afterpotentials have been shown by LORENTE DE NÓ (1947) to be produced in his so-called L fraction of the membrane potential. It is therefore very probable that most of the thermopotentials are due to changes in the L fraction and that the repolarization seen in fig. 3 is a repolarization of the L fraction. LORENTE DE NÓ has stated that depolarization of the L fraction in-itself never can initiate impulses; for this depolarization of the Q fraction is necessary. Variations in the membrane potential may be a variation of the L fraction and thus give no information about the potential change necessary for impulse generation. Our experiment actually shows that the membrane can undergo repolarization of the L fraction at the time when a discharge is initiated.

C. v. EULER found that local heating to  $45^\circ$  sets up impulses in thin myelinated and unmyelinated fibres. As an indicator of the discharge he used the blood pressure response of the animal. L7 roots, cut peripherally, were used and the blood pressure was

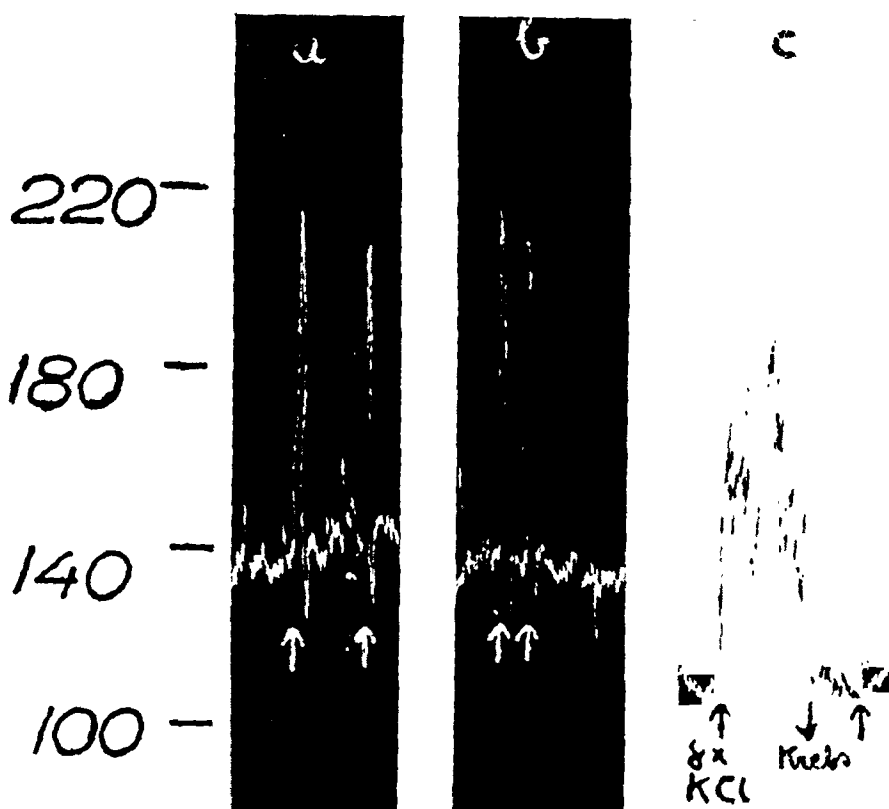


Fig. 4. Changes of blood pressure following heating to  $45^{\circ}$  of L7 dorsal root, cat. a. normal root. b. After treatment of the root with Krebs solution with 5 times increase. c shows the blood pressure response on treatment of the same root with a Krebs solution with 8 times KCl.

measured in the common carotid artery. Heating to  $45^{\circ}$  gave a blood pressure rise amounting to 50–100 mm Hg. It started when the temperature of the thermode was above  $40$ – $44^{\circ}$ . The discharge was not much dependent upon the rate of warming of the root, it depended rather upon the final temperature reached. Confirming EULER we never saw cooling cause a rise of blood pressure but, sometimes, when the temperature was returned to  $37^{\circ}$  there was a rise of blood pressure indicating a discharge in the thin fibres.

In view of the present problem we are faced with the question as to whether treatment with excess potassium of C fibres is going to change the discharge to heating in the same way as it changes the effect of temperature on the membrane- (and on the action-) potential. The roots used for experiments on whole animals may not always be in the same state as in the excised condition be-



cause some vascularization must remain and counteract the effect of potassium. But a progressive rise of the potassium concentration must sooner or later reproduce the conditions obtained with excised nerve. Treatment of a root with Krebs solution in which the concentration of potassium was increased did not diminish the blood pressure rise to heating. Fig. 4 a demonstrates a normal blood pressure rise following heating to  $45^\circ$  of a L7 sensory root. Fig. 4 b shows that the response is not altered after treatment with Krebs solution containing 5 times the normal amount of potassium. Potassium above 6 times the normal concentration produces a blood pressure rise even at  $37^\circ$ , which continues as long as the solution is allowed to act. Fig. 4 c demonstrates this stimulation by excess potassium. It is seen that washing with normal Krebs immediately abolishes the discharge.

These experiments therefore have shown that although effects of temperature upon the membrane potential and action potential in A and C fibres can be compensated by variations in the concentration of potassium ions in the external medium, the selectivity of the thermal mode of stimulation nevertheless persists, the thin fibres, still being excited by heat, the thick myelinated ones by cooling.

II. On the property of potassium-free solution to synchronize a massed discharge in the excised motor root.

In previous experiments concerning the temperature sensitivity of the membrane potential it was observed that the discharge on cooling differed much if the root was treated with Ca-free solution or with a solution lacking both  $Ca^{++}$  and  $K^+$ , and that observation led us to make the experiments reported here.

As has been described earlier in this paper cooling cannot elicit a discharge in a motor root that has been excised and left in Krebs solution for some hours. The experiment illustrated in fig. 5 was made with such a root which was then treated with Krebs solution lacking  $Ca^{++}$ . As seen in record a, this caused a cold discharge to reappear. Afterwards that part of the root that was within the thermode was treated with a Krebs solution that was lacking both  $Ca^{++}$  and  $K^+$ . Record b shows that a discharge appeared which at  $25^\circ$  waxed and waned at intervals of about 10 msec. Record c was taken shortly after record b at a slower sweep speed and demonstrates that the height of the fast deflections, shown in record b, was modified by a slow rhythm so that at  $25^\circ$  a maximum appeared at every 140 msec. This synchroniza-

sodium phosphate to pH 7.3 in exchange for sodium chloride of the Krebs solution). It has been shown by MONNIER (1946) that phosphate ions in frog nerve create a discharge that often is rhythmic. Treatment with the concentration mentioned above

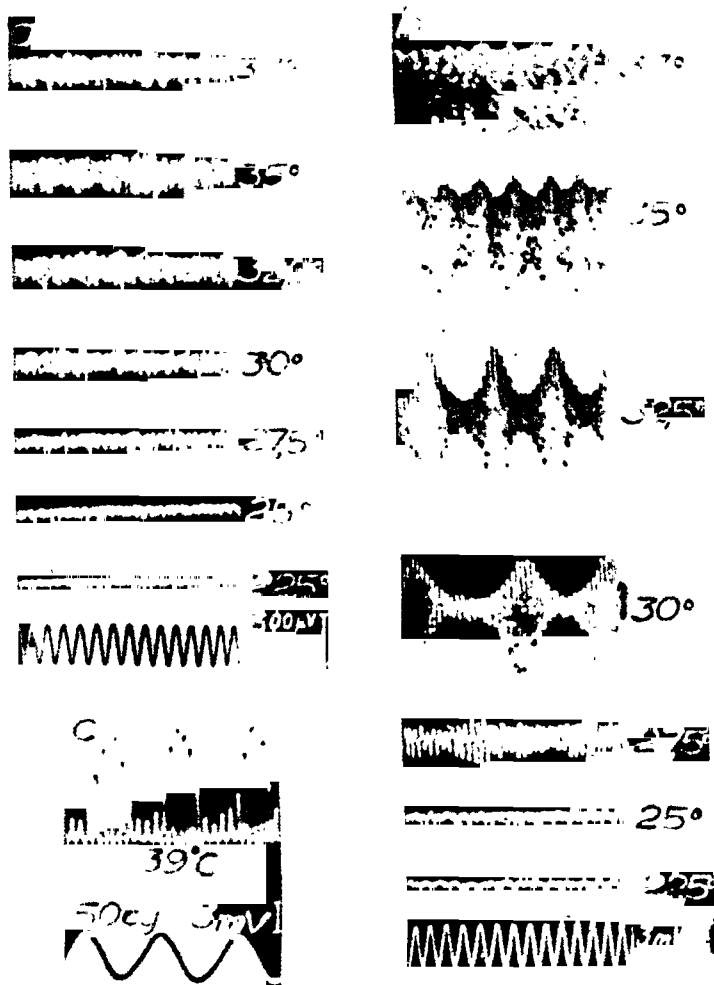


Fig. 6. Motor S1 root. cat. a. After treatment with Krebs solution containing 0.03 M sodium phosphate. b. The same root after 5 minutes treatment with a solution containing the same amount of phosphate ions but no potassium. c. A root from another experiment after the same treatment as in fig. b.

gives in motor roots a spontaneous discharge maximal between 40° and 35° and cooling causes a reduction of that discharge (fig. 6 a). Fig. 6 b demonstrates how this discharge was changed after 5 min. treatment with a solution that contained the same concentration of phosphate ions without potassium. The discharge appears in volleys at every 4 msec. (37°) and the height of these

and ERLANGER, 1930, H. T. GRAHAM, 1934) which in turn are dependent upon the L fraction of the membrane potential (LORENTE DE NÓ, 1947). But since the L fraction together with the phenomena dependent upon it are very apt to exhibit slow fluctuations (LORENTE DE NÓ, 1947, GASSER and GRUNDFEST, 1936, LEHMANN, 1937) it seems plausible to suppose that the slow oscillations of response height reflect the simultaneous fluctuation of the L fraction.

The effect of removal of potassium from a Krebs solution already poor in Ca or containing phosphate therefore seems twofold. First it increases the interaction between fibres so that discharges are synchronized. Second it causes a fluctuation of the L fraction so that excitability undergoes a periodic variation.

### Summary.

1. It was previously known
  - a) that A fibres are excited by cooling and C fibres by heating.
  - b) that the nerve membrane potential of A. fibres is maximal at  $37^\circ$ , but with C fibres the maximum is at  $25^\circ$  (fig. 1).
  - c) that the effect of low  $K^+$  upon the A fibres is to make the temperature potential curve resemble that of C fibres; the effect of high potassium upon C is to make them resemble A in this respect.

The object of this paper is to see whether the differences in the action of temperature changes upon A and C (a above) is reversed when by removing  $K^+$  from A or adding it to C their temperature-potential relations have been interchanged.

2. Using the spinal roots of cats it was found that A fibres are still excited by cooling and C fibres by warming. This lends no support to the belief that temperature excitation is directly due to the local differences in membrane potential.

3. When  $Ca^{++}$  and  $K^+$  were removed from the A fibres which were then cooled a synchronous discharge occurred at 100/sec. at  $25^\circ$ . The amplitude showed a regular fluctuation at 7/sec.

4. The same double rhythm was obtained with  $K^+$ -free nerves discharging spontaneously due to excess phosphate ions. The slow amplitude wave has a temperature coefficient  $Q_{10}$  of about 10.

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## The Effect of Salyrgan (Mersalyl) on the Osmotic Pressure of the Blood.

By

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Received 31 December 1948.

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That mercurial diuretics have a renal action is generally accepted, and the probable existence of an extrarenal effect has been emphasized by several authors. The latter property of the mercurial diuretics is suggested by significant changes in the chemical composition of the blood (for references see below) as well as by altered absorptive properties of the dermal connective tissue (DONATH and TANNE 1927, ENGEL and EPSTEIN 1931). In salyrgan-treated nephrectomized rabbits MÖLLER (1930) observed a decreased concentration of hemoglobin. This was interpreted as as being due to a shift of fluid rich in chlorides to the blood, resulting in hydremia. Individuals with intact kidneys have also exhibited an initially increased chloride level during the first phase of the action of mercurial diuretics (BOHN 1923, SAXL and HEILIG 1923, DECOURT et al. 1936).

A capacity for storing chloride has been attributed to the skin and there is some evidence to support the hypothesis that, at least temporarily, the skin can store sodium chloride "dry", *i. e.* with insufficient water to form an isotonic solution (PADT-BERG 1910). More recent studies (OTTOSEN 1945) indicate that the skin may store sodium in the same way. So stored, osmotically hypertonic tissue fluid, rich in sodium chloride, may be diverted from the tissues (dermal) to the blood.

In a previous report (EDLUND and LINDERHOLM 1947) an extrarenal effect was demonstrated during the first hour following the administration of salyrgan. The absorption of water and col-

loid (crude human hemoglobin) from the knee joints of urethaned rabbits was, thus, significantly greater than that of untreated controls. No increase in diuresis was found during the same period. An increase in the total osmotic pressure of the blood was put forward as one possible causal mechanism of this effect.

It is not *a priori* impossible that salyrgan may cause a raised total osmotic pressure of the blood by mobilizing stored electrolytes. Such an action of the drug would be a parallel to the one found by BARBOUR (1932) in perorally waterloaded rabbits after administration of pituitrin. He determined the osmotic pressure of the blood by a method similar to that used in the present investigation and came to the conclusion that pituitrin transfers a slightly hypertonic fluid from the tissues to the blood. However, since he disregarded the possibility of the glandular extract causing an altered absorption of water from the digestive tract, his experiments and conclusions are open to criticism.

The osmotic pressure of human blood following salyrgan treatment has previously been determined by a method of satisfactory accuracy (BLEGEN 1940); after salyrgan treatment there was no significant change. The experiments were, however, performed on edematous subjects with good diuretic response thereby possibly concealing any changes in the osmotic pressure caused by an extrarenal action of the drug.

### Methods.

Male rabbits weighing  $2.1 \pm 0.1$  kg were used. They were fed hay, oats and water *ad lib*. Anesthesia: 6 ml per kg of body weight of 25 per cent urethane solution was injected intravenously during 20 minutes. 30 minutes after the end of the urethane injection 2–3 ml of arterial blood were withdrawn by heart puncture and collected under paraffin oil in a centrifuge tube containing 0.5 mg dry heparin. (The amount of heparin used had no influence on the osmotic pressure.) The blood was at once centrifuged and the plasma drawn into small all-glass syringes for the determinations of the osmotic pressure. The syringes were fitted with needles of very small internal diameter which were carefully wiped on filter paper to prevent any adherent fluid from drying on the needle tip. To prevent evaporation from the contents of the syringes the plungers were greased with high-grade vaselin.

In the same way blood samples were taken 30, 60 and 90 minutes after the first specimen and treated as described above. Immediately after the first blood sample was drawn 6 rabbits were given 5 mg of salyrgan per kg of body weight as a 1 per cent solution intravenously. 6 untreated controls were also used. The body temperature was held within normal limits by means of an electrically heated operating table.

$$f_1(t) = \frac{1}{2} \left( 1 + \frac{1}{2} \cos \frac{2\pi t}{T} \right) \quad (1)$$
[illegible][illegible]

from joint cavities following salyrgan treatment. The present material exhibited no such increase in the total osmotic pressure of the blood from salyrgan-treated animals. Consequently the increased absorption of water and colloid from the joint cavities after salyrgan treatment of an experimental material equivalent to that used for the present investigation cannot be due to a rise in the osmotic pressure of the blood.

The reduced osmotic pressure noted in some animals during the experimental period, which reduction was significantly in excess of the error in the osmotic pressure determinations, is difficult to explain. It may be due to some side-effect of the anaesthetic.

### Summary.

Determinations of the total osmotic pressure of rabbit blood plasma after treatment with salyrgan showed that the drug was without effect in this respect during the hours immediately following administration. The action of salyrgan<sup>1</sup> on absorption from joints, found by the authors in an equivalent material, cannot, therefore, be due to a rise in the osmotic pressure of the blood.

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<sup>1</sup> The salyrgan was obtained by courtesy of AB Leo, Hålsingborg.



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## The Action of Salyrgan on Skin Permeability — Salyrgan as a Spreading Factor.

By

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Received 31 December 1948.

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Among described extrarenal effects following the administration of mercurial diuretics these may be mentioned: 1) a more rapid disappearance in non-edematous human subjects of intracutaneously injected saline wheals (ENGEL and EPSTEIN 1931), 2) subcutaneously injected sodium fluoresceinate appears in the blood earlier (DONATH and TANNE 1927), 3) an increased flow of edema fluid from Curshmann needles inserted in edematous tissue (HOFF 1925, TSCHERNING 1927, OFFENBACH 1928) and 4) an increased absorption of colloid (hemoglobin) and fluid from the knee joints of salyrgan-treated rabbits (EDLUND and LINDERHOLM 1947). The last mentioned effect of salyrgan could possibly, as previously suggested (EDLUND and LINDERHOLM 1947), be ascribed to a supposed action of the drug on connective tissue similar to that of "spreading factors". (Cf. DURAN-REYNALS 1942 and MEYER 1947 for bibliography on "spreading factors".)

To put this hypothesis to the test the following experiments were devised.

### Method.

A slight modification of Madinaveitia's method was used to investigate the spreading factor activity of intravenously and locally injected salyrgan.

### Isotonic Hemoglobin Solution.

An isotonic solution of 7 gram per cent of hemoglobin (human) was prepared as follows: 2 grammes of sodium chloride were added per 100 ml of the hemolysate of three times washed corpuscles hemolyzed with 2 volumes water. The ghosts of the corpuscles were then removed by centrifuging. After dialysis and dilution to adequate strength the solution was made isotonic by the addition of sodium chloride and 10 mg per cent of calcium ( $\text{CaCl}_2$ ). The solution was sterilized by filtering through a Seitz' filter.

### Injections.

Male albino rabbits weighing  $2.0 \pm 0.1$  kg were used. A paste containing 100 g BaS, 70 g ZnO and 120 g wheat starch was used to depilate their backs and flanks 24 hours before the tests. Animals exhibiting signs of dermal irritation following the depilation were rejected.

About 2 cm from a line drawn along the backbone 3 to 5 symmetrical points were marked on each flank. 0.3 ml of the hemoglobin solution was injected at each point so that the centre of the wheal coincided with the point. The injections having been made on one side of the line (control side) the area of each wheal was measured 4 times, 60—68 minutes after the injection.

The animals to be tested with salyrgan were then given an intravenous injection of the drug (5 mg per kg of body weight of a 1 per cent solution). Thereafter the hemoglobin solution was injected on the other side of the line (test side). The areas of the wheals were then measured as described above. In another group of animals hemoglobin solution only was injected on one side (control side) and the same hemoglobin solution containing 10 mg per cent of salyrgan was injected on the other side (test side). The injections on each side were made consecutively with an interval of 10 minutes between the sides. The "test side" and the "control side" were alternately injected first. The areas were then measured after the same elapse of time as before.

In a third group of animals the rabbits were first killed by a blow on the neck. 15 minutes after the cessation of the heart beats the animals were treated like those given salyrgan locally. Readings in this group were taken after 10 and 60 minutes.

In order to ascertain whether the injections of hemoglobin on one side of the backbone could influence the area of spreading of similar injections performed about an hour later on the other side, a series of animals were given hemoglobin injections in the skin just like the animals given salyrgan by the intravenous route save that the drug was omitted. This procedure simultaneously affords a good measure of the reliability of the method adopted.

### Measurement of the Areas.

The rabbits were placed in a box with their necks fixed in an aperture (see ill.). The longest and shortest diameters of the wheals were meas-



ured with calipers at the four different times as described above. The areas were calculated on the assumption that the coloured patches were regular ellipses (Madinaveitia).

The mean area on each side was then calculated and the difference between the sides was treated as a statistical unit.<sup>1</sup> In the subsequent statistical calculations the differences were weighted in proportion to the number of symmetrical patches on each animal.

### Results.

The results are given in table 1.

Table 1.

	Time in minutes after intrader- mal injection	Mean area of spreading in mm <sup>2</sup> .		Mean difference $\pm$ standard error of the mean
		Test side	Control side	
Salyrgan intravenously. 12 animals .....	60—68	375	315	$60 \pm 10.5$ $P < 0.001$
Salyrgan locally. 11 animals	60—68	378	332	$46 \pm 7.0$ $P < 0.001$
Dead animals. 9 animals...	10	283	244	$39 \pm 9.6$ $P < 0.01$
„ „ „ „ „ .....	60	369	322	$47 \pm 10.8$ $P < 0.01$
Control animals. (No sal- yrgan.) 10 animals .....	60—68	338	334	$4 \pm 6.3$ $P > 0.5$

P = Probability that the difference is caused by random elements.

<sup>1</sup> The statistical calculations were made according to the formulas for small samples as given in Fischers Statistical Methods for Research Workers.

From the table it is evident that the intravenously and locally injected salyrgan has an action similar to that of spreading factors. The same effect was observed in dead animals treated locally. The differences between the sides are statistically significant. From the untreated control animals it appeared that the spreading in the side treated last remains uninfluenced by injections of hemoglobin about an hour earlier in the opposite side.

### Discussion.

From the results it is evident that salyrgan acts on the permeability of the skin in the same manner as a spreading factor of low activity. The objection may be raised against the local application of salyrgan in experiments on living animals that the increased spreading of the hemoglobin in these animals might have been caused by an inflammatory action of salyrgan on the tissues. Increased spreading in states of early inflammation has been reported by McMASTER and PARSONS (1939) and HECHTER (1947). However, no macroscopical signs of dermal inflammation were observed in connection with our experiments. Furthermore, the experiments on dead animals showed that the increased spreading caused by salyrgan is independent of any circulatory or inflammatory provocative action of the drug.

Further evidence that some of the extrarenal actions of salyrgan may be explained by a spreading factor activity of the drug are given below.

ENGEL and EPSTEIN (1931) demonstrated that treatment with mercurial diuretics lessens the disappearance time of subcutaneously injected saline wheals in non-edematous human subjects. In several respects the wheal disappearance test of ALDRICH and McCLURE, adopted by ENGEL and EPSTEIN, is, however, equivalent to a measure of tissue permeability (DURAN-REYNALS 1942). Consequently the results of ENGEL and EPSTEIN corroborate the findings presented in the present paper.

In the opinion of the present authors there is, however, no valid substantiation for an absolute correlation between the spreading phenomenon and the saline wheal disappearance time. An increased absorption of fluid or coloured matter via the blood or lymph capillaries may occur without any concomitant increase in the permeability of connective tissue. With dyes as indicators this has been found to be the case in respect of inflamed skin (HUDDACK and McMASTER 1933, MILLER 1938).

It has been shown, furthermore, that subcutaneously injected sodium fluoresceinate appears more rapidly in the blood after treatment with mercurial diuretics (DONATH and TANNE 1927). It is known that local treatment with spreading factors increases the absorption to the blood of substances injected in the treated area. (McCLEAN et al. 1933, 1934, SAMMARTINO 1937, cf. DURAN-REYNALS 1942.)

The increased absorption of hemoglobin and fluid from knee joints of salyrgan-treated rabbits is not caused by any change in the osmotic properties of the blood (EDLUND and LINDERHOLM, this journal in press), but may be explained by an increased permeability in the synovial membrane.

Typical of the action of spreading factors is their reduction of the resistance to flow in connective tissue.

McMASTER (1941) has demonstrated that the infusion rate of Lockes' solution in dermal connective tissue of the mouse under varying hydrostatic pressures is relatively constant for low pressures, but rises markedly, and is directly proportional to the pressure for pressures above the "breaking point", the mean value of which corresponds to 8.5 cm of water. No such "breaking point" exists in edematous tissue where the homogeneity of the interfibrillar matrix of the connective tissue is interrupted by free fluid (McMASTER 1941). For all used pressures there is in edematous tissue, direct proportionality between the pressure and the infusion rate.

A method similar to that developed by McMASTER was used with respect to knee joints of rabbits and thereby it was possible to verify the existence of a "breaking point" for the synovial membrane also. Furthermore, this "breaking point" is of the same magnitude as that found by McMASTER.<sup>1</sup> In most cases the "breaking point" vanishes after intravenous injection of salyrgan; indicative of the drug causing a lessened resistance to flow.<sup>1</sup>

A similar disappearance of the "breaking point" has been found after exercising the rabbits in a tread mill 16—18 hours before the infusion experiments.<sup>1</sup> Tests similar to those reported in a previous paper (EDLUND and LINDERHOLM 1947) on the absorption of a hemoglobin solution demonstrate that the absorptive changes are of the same type in the exercised and the salyrgan-treated rabbits.<sup>1</sup>

<sup>1</sup> These experiments will be described on a later occasion in papers by EDLUND, LINDERHOLM and EDLUND and LINDERHOLM.

*Most experiments cited above together with those presented in this paper seem to indicate that these extrarenal effects of salyrgan may be attributed to a spreading factor action of the drug.*

Another extrarenal effect of mercurial diuretics is the increased flow of edema fluid from Curshmann needles inserted in edematous tissue. Theoretically such increased flow could be ascribed to any factor that decreases the resistance to flow in connective tissue. However, it still remains to be proved whether the decreased resistance to flow induced by salyrgan — as measured by the spreading phenomenon in the skin or the decreased resistance to flow in the synovial membrane — has any relation to a similar effect in edematous tissue.

The mechanism of the spreading action possessed by salyrgan is not understood. The drug has no effect on the viscosity of hyaluronates or on the rate of viscosity change of mixed hyaluronidase and hyaluronates.<sup>1</sup>

Experiments with methods similar to those adopted by MAYER et al. (1948) are in progress with respect to the effect of the drug on the amount of free hyaluronidase in the skin.<sup>1</sup>

### Summary.

A spreading factor activity of intravenously and locally injected salyrgan<sup>2</sup> was demonstrated. The increased spreading was also exhibited by dead, locally treated, rabbits up to about 1 hour after death.

The spreading factor activity of salyrgan is discussed in relation to some of the extrarenal effects of the drug, and it was concluded that most of these effects may be attributed to a spreading activity of the drug.

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## **Some Observations Concerning the Pathophysiological Effects on the Human Skin Caused by the Stinging Jellyfish (*Cyanea Capillata*).**

By

ANDERS KRISTENSON.

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During the summer months jellyfish are met more or less abundantly on the west coast of Sweden. These creatures belong to the cavity invertebrates or coelenterata, and are represented by many species. One of these, which is commonly called the stinging jellyfish (*Cyanea capillata*), is characterized by the fact that its tentacles are provided with stinging threads or cilia and cause a prolonged smarting sensation when they come into contact with the human skin.

### **Pathophysiological Effects.**

When the stinging jellyfish's threads come into contact with a skin the horny layer of which is thin, it usually gives rise to a practically immediate intense burning sensation. This is either felt at a certain point or radiates somewhat into the surrounding area. No sensation of unspecific warmth or cold or actual pain is felt, but only a slight, unspecific sensation of contact. The effect appears to be more intense and immediate if the tentacles are drawn over the skin. If the tentacle is laid carefully on the skin, there may be no stinging effect. On the other hand, if the tentacles are pressed into closer contact with the skin, or if they are rubbed against the skin, the effect is quicker. When the tentacles are removed from the skin after the sting has been felt, a more or less intense and protracted burning sensation persists.



This varies in intensity; it comes and goes in different places, now as a single burning spot, now as a radiating, smarting sensation. The sensation seems to be felt spontaneously and continues with diminishing intensity for some time, even for about twelve hours afterwards. During the first few hours the smart is fairly constant, even though it may be felt in different parts of the affected area. Later the pain may disappear for increasingly long periods, only to return suddenly without any obvious reason, spontaneously as it were.

Within the area directly affected slightly reddened patches appear, and these may persist for a short time. A certain degree of oedema can also be observed in some cases, although it is always very slight. I have not observed any definite effect on the capillaries or other vessels. No irritation of the lymphatic vessels or lymph glands has been noticed.

Certain stinging jellyfish, which I believe I have found to be especially the more markedly red ones, have a more severe effect than the others. Some of those which have most effect also act on the pilomotor muscles, which become spastically contracted, and give rise to typical goose-flesh. This phenomenon persists for several hours after contact with the tentacles. Subsequently an increased irritability of the pilomotor muscles may persist, and this manifests itself in a strong, and sometimes very sustained contraction after the area has been lightly stroked with a finger or other object. The effect on the pilomotor muscles is not confined solely to the area touched by the tentacles, but gradually appears in the surrounding area also, especially at the sides and centrally, apparently owing to absorption of the active substance through the lymphatic vessels. This pilomotor spasm and irritability of the pilomotors is not associated with any appreciable sensation.

There seems to be no evident effect on the sense of touch, but if the affected area is touched, at least during the first few hours a clear increase in the burning sensation follows. The sense of pain is not demonstrably affected.

A peculiar phenomenon presents itself at the warm and cold spots, especially at the latter. If, before applying the tentacles, one marks the cold and warm spots on the skin, it is found that it is precisely at these places that the stinging sensation is felt. If these points are stimulated with heat and cold respectively after a sting, one finds that the sense of heat and cold is practic-

ally unaffected. However after a short latent period at first only a fraction of a second but later some seconds a fairly intense burning sensation is felt, even following an insignificant cold or warm stimulus. The pain is sometimes strictly confined to the stimulated spot, sometimes it radiates over a larger area. This sensation can also be elicited without touching the skin with cold or warm objects; just blowing lightly on the skin evokes a burning sensation. This can occur without preceding or accompanying hot or cold sensations. It is easy to be convinced that it is a question of the same sensations after the sting of a jellyfish as after stimulation from hot objects. Subjectively the sensation is the same in both cases. A slight stimulation, neutral as regards temperature, of the cold and warm spots has no effect, neither has cold or warm stimulation between the spots. These conditions continue for a long time, even for more than 24 hours. Then, when the spontaneous burning sensation has disappeared, by stimulating the cold, and also, to a lesser degree, the warm spots with moderate intensity, one can evoke a burning sensation, although it is less marked than before.

Here it is a matter of a specific effect of the toxin of the jellyfish on a special organ situated in the warm and cold spots for the perception of the sensation: burning. This organ is very greatly sensitized by the toxin. Topographically-anatomically it appears to be intimately associated with the organs for temperature perception. But functionally it can be differentiated from these organs. The normal specific stimulus is heat, but after stimulation with jellyfish toxin it reacts to very slight temperature stimuli also. When the warm spots are stimulated with cold and the cold spots with heat, the sensitized organ reacts with its burning sensation, even without the occurrence of cold or warm sensations.

The following phenomenon is also of interest: if there is a stimulation of the cold spots central to the affected area, but outside the area which is influenced by the absorbed toxin, a normal sensation of cold results. After a latent period of some seconds, however, at some other point in the affected area there is a burning sensation of the same type as that supervening on direct stimulation of the sensitized cold spot, but it is less intense. The interpretation of this state of things is a hazardous matter, but it might point to an absorption effect on a considerable nerve area after local application.

If jellyfish tentacles are placed on an area of the skin which is free from warm and cold spots, no immediate effect results. It is several minutes before, first an insignificant, and then an increasingly strong burning sensation follows. At first this sensation appears in the temperature spots which lie nearest the point of application, later to occur at points lying further out. If the application is made directly on the warm and cold spots, a practically immediate effect is obtained. This fact can be established by taking the same tentacle and moving it from an area without temperature spots to one with them. After the application of tentacles to an area without temperature spots, the temperature spots surrounding this area are found, on examination, to be sensitized before spontaneous burning sensation are felt.

From individual experiments it appears that there are spots where there is no temperature perception, but where the tentacles cause both spontaneous smarting and lasting hypersensitivity to temperature stimulation. Such spots are very rare, but they show that this toxin-affected organ is not necessarily connected with the temperature points, but can occur independently of them.

By the application of chloroform to an area of the skin it can be made anesthetic to cold for a considerable period. The perception of heat, on the other hand, does not appear to be much affected. However the cold spots which are anesthetic to cold retain their ability to react normally to heat. Consequently chloroform can be used to differentiate cold perception from heat perception. If an area that has been sensitized with jellyfish toxin is treated with chloroform, cold perception is also eliminated, but stimulation with heat or cold elicits the same burning sensation in these cold-anesthetized spots, as that which in areas affected by jellyfish toxin, but not treated with chloroform. To eliminate the possible source of error due to the fact that chloroform prevents the sensation of cold by chilling the skin, I have performed the experiment with chloroform warmed up to about body temperature. It is also possible to produce a burning sensation within this chloroform treated area merely by bringing a warm or a cold object in proximity to it. Consequently chloroform eliminates the sensation of cold but leaves both normal heat perception and toxin-sensitized perception of heat unaffected. With protracted treatment with chloroform the burning sensation possibly decreases, but it never disappears.

According to prevalent conception, as set forth in text books on physiology (STARLING, HÖBER), the skin has a sense of cold and of warmth sensations of touch and of pain, perhaps also a sense of pressure. According to STARLING the sense of heat is assumed to be due to a simultaneous stimulation of warm and cold points. No special organ for the sense of heat is thought to exist. HÖBER states that the sensation "heat" is caused by non-specific stimulation. He refers to investigations by ALRUTZ and THUNBERG, which are said to show that heat is a complex of warmth and cold.

These investigations indicate that there is a special organ in the skin which brings about this burning sensation which is caused in the case of heat. Jellyfish toxin causes this organ to become very hypersensitive, so that it reacts with a strong burning sensation to stimulation of normal temperature. Jellyfish toxin, on the contrary, does not noticeably affect the skin's perception for the rest. By means of chloroform one can eliminate the sensation of cold at the cold points, but the perception of heat and hypersensitivity to temperature stimuli caused by jellyfish toxin persists unchanged.

The circumstances advanced above afford a clearer insight into the sensitive effect on the skin caused by the stinging jellyfish. The great sensitivation of the perception of heat has the result that the least change of temperature in the affected area too gives rise to a burning sensation. When this sensation seemingly arises spontaneously, this will be due to slight changes of temperature in the skin, which do not lead to either cold or warm sensations, but only affect the hypersensitive terminal organs for heat perception. Draughts, the light touch of clothing etc. are such temperature-changing factors. It will not be denied, however, that a quite spontaneous burning sensation may also be met with. Possibly, if the jellyfish toxin penetrated a larger skin surface, a more general sensitivity might arise owing to absorbed toxin. The general malaise which is said to follow on a more extensive contact with jellyfish may perhaps be explained in this way.

### Summary.

It appears that there is a special organ in the skin which gives rise to this burning sensation, heat, and which as a rule is topographically-anatomically closely connected with the organs of

perception of warmth and cold. The toxin from the active tentacles of the so-called stinging jellyfish (*Cyanea capillata*) causes hypersensitivity in this organ, but does not noticeably affect other skin sensations. When this organ is hypersensitive owing to the effect of jellyfish toxin, it reacts to inconsiderable variations in temperature for 12—24 hours afterwards. Chloroform can eliminate the perception of cold, but the normal sensation of heat, and sensations of heat arising from small changes in temperature, when the organ is stimulated by jellyfish toxin, remain. In some cases the toxin also has a local effect on the pilomotor muscles manifesting itself in their spastic contraction. This effect of the toxin explains the subjective sensations after contact with the jellyfish.

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# Citric Acid in Calcareous Depositions and Similar Formations in Non-Vertebrates.

By

Professor T. THUNBERG, Lund.

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Since Dickens and the present author first demonstrated the high Ci-content (Ci = citric acid or citrate) of the bone substance, citric acid in fairly high concentrations has been found in calcareous depositions, especially in those also containing calcium phosphate. All findings reported, however, concerned vertebrates. It seemed to be of interest to study the occurrence of Ci in the non-vertebrates as well, mainly on account of the abundance and peculiarity of the lime concrement found in this division. According to WINTERSTEIN such concrement can be found in the Annelids, Cephalopods, Cestoids, Gastropods, Lamellibranchiates, Nematodes, and Trematodes. The surprisingly high concentration of calcium carbonate in the secretion from the oesophageal glands of the earthworm should be especially mentioned. DARWIN characterized these glands as "highly remarkable" "for nothing like them is known in any other animal". (Naturally one must bear in mind that DARWIN's statement was made in 1881, Cf. also HARRINGTON.)

This report, however, is limited to a few non-vertebrate drugs, once of special interest as medicinal treasures of older pharmacopeiae. They are as follows:

*Lapis cancri*,

*Os sepiae*.

*Lapis cancri* (often called "Lapilli cancri", or, in popular idiom "crayfish eyes") are button-shaped formations, two in number,

which appear in the two pockets of the so-called abdominal wall of the crayfish. Owing to the place of formation HUXLEY called them gastrolites. They consist of 63 % calcium carbonate, 19 % calcium phosphate, organic substance, and water (DULK). A gastrolite may weigh as much as a few decigrams. During the time of formation they are isolated in their pockets from the rest of the stomach. When the crayfish begins to change his shell the barrier between the pockets is broken, the stones emerge into the stomach where they are decomposed and resorbed. It was formerly thought that the dissolution was due to some acid in the stomach. It is doubtful, however, whether the content of the stomach is acidic.

It seems probable that the gastrolites, or rather the processes which cause the formation and dissolution of the gastrolites, play some rôle in the change of the shell. Their formation a few weeks before the crayfish sheds its shell signifies a decalcification of the old shell, so that the shell becomes more elastic, and the shedding process easier. The re-infiltration of calcium in the chitin shell implies that the shell resumes its mechanical protective rôle. — It is a well known fact that even the blood of the crayfish periodically contains calcium in a comparatively high concentration. It seems reasonable that a high calcium concentration in the blood corresponds to the decomposition of the crayfish stones and that the formation of the stones is reflected by a low calcium concentration in the blood. This problem has not been investigated, however.

*Os sepiae.* *Os sepiae* is perhaps the most common denomination of the subcutaneous cuttle-bone of the cuttle-fish, *Sepia officinalis*. After death the cuttle-fish is rapidly decomposed and the freed cuttle-bone is washed up on the shore. According to popular opinion the cuttle-bone was a scale of some kind of fish, and the old name of the drug was, therefore, "*Os pisci*". Such large scales were thought to come from the whale, the largest "fish" known, and hence the name of whale-scale.

*Methods.* In the investigations reported in this paper the method recommended by HUNTER and LELOIR was used except in the earlier experiments where the method worked out by PUCHER, SHERMAN, and VICKERY dominated. The two methods, however, have not been sharply separated. Various combinations between them have been necessary on account of prevailing circumstances. Sometimes a reagent was unsatisfactorily pure, or was impossible to obtain. The many

modifications of these methods which are still in vogue should probably be interpreted as an expression of the fact that as yet no completely satisfactory method has been found.

*Results.* The citric acid values obtained are expressed in parts per million (p. p. m.).

*Lapis cancri:* 12 determinations. Average = 8,100 p. p. m. Dispersion = 1,425 p. p. m. The difference between 8,100 and zero is most significant as it is 20 times the average deviation.

*Os sepiae:* 12 determinations. Average = 123 p. p. m. Dispersion = 102 p. p. m. The difference between 123 and zero must be considered as significant as it is 4.2 times the average deviation.

### Discussion.

The present investigation was carried out mainly to test the hypothesis that in the cases where calcium phosphate is a constituent citric acid can also be found in considerable concentration. With "a considerable concentration" is meant a concentration which surpasses the average value of the citric acid present in the tissues and fluids. The marked citric acid content cannot then be a result of diffusion processes, but must be caused by, for instance, chemical precipitating processes. The citric acid content found in the formations studied confirms the hypothesis as to the processes which have caused the concentration of citric acid. Calcium phosphate and citric acid are apparently substances that contribute to the formation of the concrements discussed in this paper.

### Summary.

Citric acid, which is a metabolite regularly occurring in the tissues and fluids of the vertebrates, has also been found in certain non-vertebrates and in larger concentrations, particularly in concrements containing calcium phosphate. Citric acid can be demonstrated in crayfish stones in an average concentration of 8,100 p. p. m. and in *os sepiae* in an average concentration of 123 p. p. m.

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The author is indepted to Amanuens JOHANNESSEN and to Miss BIRGIT LUNDQVIST for good help.

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## **Influence of two different Fats on reproduction Capacity of Vitamin E Deficient Rats.**

By

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Received 8 March 1949.

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It has been demonstrated that certain symptoms in chicks, viz., exudative diathesis, brown coloration of the adipose tissue, and encephalomalacia, and in rats, viz., yellow-brown coloration of the adipose tissue, and depigmentation of the incisors, depend upon the feeding of vitamin E deficient diets containing certain highly unsaturated fatty acids (DAM 1944 a and b, DAM and GRANADOS 1945 a and b, GRANADOS and DAM 1945 a and b, GRANADOS, MASON and DAM 1945 and 1946). Formation of peroxides preceded, and in some instances paralleled, the brown coloration in the adipose tissue of these animals (DAM and GRANADOS 1945 a and b). The pigment responsible for the coloration of the adipose tissue was found to consist of at least 2 fractions: one of them is fat soluble, while the other, which occurs in larger amounts, is not extracted by lipid solvents. A preliminary chemical characterization of the latter pigment showed it to agree with the description of "ceroid", the pigment found in various tissues in experimental liver cirrhosis in rats (DAM and GRANADOS 1945 a, GYORGY and GOLDBLATT 1942, ENDICOTT and LILLIE 1944).

Histologically, the gross pigmentation in the adipose tissue was found to be represented by an acid-fast pigment in the fat cells at different stages of development. Furthermore, the histological

Table 1.

*Diets fed to the six groups in percentage of grams.*

Ingredients	Group 1 <sup>1</sup> — — fat	Group 2 <sup>1</sup> + E — fat	Group 3 — E — + cod liver oil	Group 4 + E + cod liver oil	Group 5 <sup>1</sup> — E — + lard	Group 6 <sup>1</sup> + E + + lard
Choline chloride .....	0.4	0.4	0.4	0.4	0.4	0.4
Vitamin mixture <sup>2</sup> .....	0.5	0.5	0.5	0.5	0.5	0.5
Salt mixture <sup>3</sup> .....	5.0	5.0	5.0	5.0	5.0	5.0
Crude casein .....	25.0	25.0	25.0	25.0	25.0	25.0
Sucrose .....	69.1	69.1	49.1	49.1	49.1	49.1
Cod liver oil <sup>4</sup> .....			20.0	20.0		
Lard <sup>4</sup> .....					20.0	20.0
$\alpha$ -tocopherol acetate <sup>5</sup> .....		0.020		0.020		0.020

<sup>1</sup> To each animal of these groups were given weekly 1,160 I. U. of vitamin A, and 19 chick Units of vitamin D<sub>3</sub> in 3 drops of peanut oil.<sup>4</sup>

<sup>2</sup> The 0.5 g of vitamin mixture contained biotin 20  $\mu$ g, folic acid 400  $\mu$ g, thiamine hydrochloride 5 mg, riboflavin 5 mg, pyridoxine hydrochloride 5 mg, calcium pantothenate 5 mg, nicotinic acid 7.5 mg, p-aminobenzoic acid 100 mg, inositol 100 mg, vitamin K substitute 3 mg, and sucrose 269,080 mg.

<sup>3</sup> The salt mixture used was McCollum's Salt Mixture No. 185, supplemented with 13.5 mg KJ, 139 mg CuSO<sub>4</sub>, 5H<sub>2</sub>O, and 556 mg MnSO<sub>4</sub>, 4H<sub>2</sub>O per 100 g.

<sup>4</sup> The lard and cod liver oil were added fresh every day to the basal diet.

<sup>5</sup> We thank F. Hoffman-La Roche & Co., Basle, Switzerland, for the kind supply of the  $\alpha$ -tocopherol acetate (Ephynal) used in these studies.

age daily weight increase of which was used as a control for the daily weight increase of the mated animals.

A resorption gestation was recorded when a positive "placental sign" was accompanied by a higher weight increase as compared with the average weight gains of the 3 controls, followed by weight loss. Likewise, an implantation failure was recorded when the "placental sign" was consistently absent, and the weight increase was the same as that of the unmated controls. Furthermore, the animals which showed either implantation failure or implantation resorption were killed 22 days after the mating had begun, and their uteri were examined in order to ascertain the pseudo-pregnancies that might have occurred. The females which delivered young were left with them for a week, at which time they were sacrificed. The data presented in Table 3 in-

cludes only those animals that were mated with males which proved to be fertile before and after the mating with the experimental females.

After sacrifice of the animals the color of their adipose tissue was recorded, and samples of intraperitoneal fat were taken for determination of peroxides by the method of KING, ROSCHEN and IRWIN (1933) as modified by DAM and GRANADOS (1945 a).

### Results and Discussion.

Fig. 1 shows the average growth curves of the six groups. It is obvious that groups 1 (— E — Fat) and 2 (+ E — Fat), which received no dietary fat other than three drops of peanut oil per

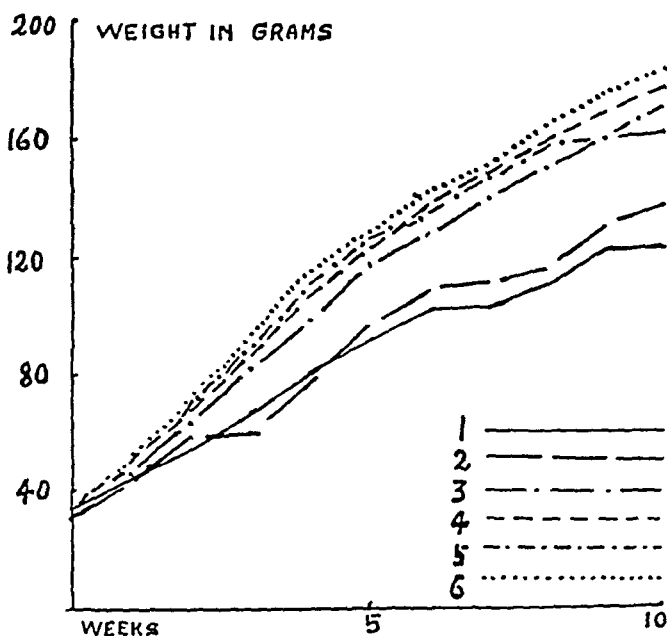


Fig. 1.

week given as a vehicle for vitamins A and D, exhibited the poorest growth. It should be observed that group 2, which received a high supply of tocopherol but without adequate amounts of dietary fat, exhibited a much lower growth rate than groups 3 and 5, which received no vitamin E but were given high amounts of fat. It is possible that the low caloric intake as well as a deficiency of essential fatty acids (the animals from groups 1 and 2 exhibited varying degrees of scaly tail), both derived from the absence of dietary fat, may have been responsible for such a poor growth. In this connection DEUEL et al. (1947) have shown the

importance of dietary fat for the normal growth and reproduction of rats.

Furthermore, comparing the growth of groups 3 (— E + cod liver oil) and 5 (— E + lard), and the growth of groups 4 (+ E + cod liver oil) and 6 (+ E + lard), it can be seen that the groups which received cod liver oil exhibited growth rates somewhat lower than the groups which were given lard. This might be explained by the toxic properties of cod liver oil.

Table 2 presents the gross discoloration and the peroxide values of the adipose tissue in the six groups. Confirming our previous observations (DAM and GRANADOS 1945 a and GRANADOS and DAM 1945 b), only group 3, *i. e.*, those animals which were given a vitamin E deficient diet containing 20 % cod liver oil, exhibited varying degrees of yellow-brown coloration of the adipose tissue. Also in these studies the subcutaneous fat appeared less coloured than the intraperitoneal depots.

Likewise, group 3<sup>1</sup> was the only one which exhibited significant peroxide values. This also agrees with our previous finding of a certain parallelism between the gross coloration of, and the peroxides found in the adipose tissue. Since group 5<sup>2</sup> (— E + lard) did not exhibit coloration of the fat concomitant with almost a complete absence of peroxides, in contrast to what was found in group 3, this supports once more the suggestions of an inter-relationship between peroxidation and the yellow-brown coloration of the fat. On the other hand, a study of the peroxide values found in the various animals of group 3 makes it apparent that the amount of peroxides present at a given time does not always parallel the degree of yellow-brown coloration of the fat tissue.

A study of table 3 makes it clear that in reproduction, as previously seen in the growth rate, groups 1 and 2 exhibited the poorest performance. Group 2, which received a high supply of tocopherol but without adequate amounts of dietary fat, exhibited a much poorer reproduction record than group 5, which received no vitamin E but was given high amounts of lard. Also here it may be possible that the low caloric intake as well as a deficiency

<sup>1</sup> This group exhibited depigmentation of their incisors, as previously reported (Granados and Dam 1945 a).

<sup>2</sup> This group did not exhibit depigmentation of their incisors, contrary to our previous findings while working in Rochester, N. Y., U. S. A. (Granados and Dam 1945 b). This may be explained by a quantitative difference of the tocopherol content in the lard of the two cities (0.8 mg per cent tocopherol in the lard used in Copenhagen), or by quantitative and/or qualitative differences in their content of highly unsaturated fatty acids.

Table 2.

*Discoloration and peroxide values of the adipose tissue in the six groups.*

Group No.	Animal No.	Discoloration of adipose tissue <sup>1</sup>		Peroxide values of intraperitoneal fat
		subcutaneous	intraperitoneal	
Group 1 — E — fat	1	—	—	0
	2	—	—	0
	3	—	—	0
	4	—	—	0
	5	—	—	0
	6	—	—	0
	7	—	—	0
	8	—	—	0
Group 2 + E — fat	9	—	—	0
	10	—	—	0
	11	—	—	0
	12	—	—	0
	13	—	—	0
	14	—	—	0
	15	—	—	0
Group 3 — E + cod liver oil	16	++	++	18.40
	17	++++	++++	26.80
	18	++++	++++	2.00
	19	+	++	0.26
	20	+	+++	6.20
	21	—	—	0.53
	22	+	++	0.50
	23	—	++	1.40
	24	++	+++	2.00
Group 4 + E + cod liver oil	25	—	—	0
	26	—	—	0
	27	—	—	0.27
	28	—	—	0
	29	—	—	0
	30	—	—	0.60
	31	—	—	0
Group 5 — E + lard	32	—	—	0
	33	—	—	0
	34	—	—	0
	35	—	—	0
	36	—	—	0
	37	—	—	0.24
Group 6 + E + lard	38	—	—	0
	39	—	—	0
	40	—	—	0
	41	—	—	0
	42	—	—	0
	43	—	—	0
	44	—	—	0

<sup>1</sup> The symbols —, +, ++, +++, +++++, and ++++++ indicate no color, pale yellow, yellow, dark yellow, yellow-brown, and dark yellow-brown color, respectively.

Table 3.

*Reproduction performance of the six groups.*

	Group 1 — —fat	Group 2 + —fat	Group 3 — + cod liver oil	Group 4 + + cod liver oil	Group 5 — + lard	Group 6 + + lard
No. of experimental animals ...	8	7	9	7	6	7
No. of rats with implantation failure .....	7	5	7	2	3	0
Percentage .....	87.5	71.5	77.8	28.6	50	0
No. of rats with resorption gestation .....	1	0	1	0	1	0
Percentage .....	12.5	0	11.1	0	16.7	0
No. of rats which delivered young	0	2	1	5	2	7
Percentage .....	0	28.5	11.1	71.4	33.3	100
Total number of young born ...	0	16	3	45	16	51
No. of still-born .....		0	2	0	2	0
Percentage .....		0	66.7	0	12.5	0
No. of young born alive .....		16	1	45	14	51
Percentage .....		100	33.3	100	87.5	100
No. of young which died within 1st week after birth .....		16	1	14	5	7
Percentage .....		100	100	31.1	35.7	13.7
No. of young alive on the 7th day after birth .....		0	0	31	9	44
Percentage .....		0	0	68.0	64.3	86.3

of essential fatty acids resulting from the almost total absence of dietary fat could account for the very poor reproduction performance of group 2. In this connection, as mentioned previously, DEUEL et al. (1947) have shown the importance of dietary fat for the normal reproduction of rats.

In table 3 one may compare the reproduction record of groups 3 (— E + cod liver oil) and 5 (— E + lard). It is apparent that the animals which received the vitamin E deficient diet containing cod liver oil exhibited a reproduction performance much poorer than those rats which were given lard. The differences are very significant.

Table 3 shows, furthermore, that the number of implantation failures is a factor which should be very carefully considered in the evaluation of sterility in vitamin E deficient rats. Actually, table 3 demonstrates that the number of implantation failures may be much higher than that of resorption gestations. This agrees with the findings of KAUNITZ and SLANETZ (1948): vitamin E is essential for the rat to become pregnant.

Thus, these studies show once more that in vitamin E deficient rats the degree of sterility can be influenced by the kind of dietary fat. Furthermore, certain facts may be correlated: group 3 (— E + cod liver oil) had a high degree of sterility, and exhibited coloration and peroxidation of the adipose tissue, whereas group 5 (— E + lard) had a lower degree of sterility, and did not exhibit coloration or peroxidation of the adipose tissue. Thus the highly unsaturated fatty acids of cod liver oil may have been responsible for aggravating the sterility symptoms of vitamin E deficiency to a larger extent than the fatty acids of lard, as is known to be the case with the changes in the adipose tissue. Therefore it can be suggested that the peroxidation of certain unsaturated fatty acids, due to the absence of the antioxidant effect of vitamin E, may play an important rôle in the development of sterility. In this connection it should be mentioned that even in the early studies on vitamin E several workers found that sterility could be produced more readily by diets containing considerable amounts of certain fats than by means of rations containing small amounts of fat. Furthermore, marked qualitative differences were noted among the various fats in their ability to induce sterility. This subject has been recently reviewed by MASON and HARRIS (1947).

In the present studies in groups 1 and 2 dietary fat was rigidly excluded, a fact which complicated the vitamin E deficiency with a deficiency of caloric intake and of essential fatty acids. It might well be that rats on a vitamin E deficient diet containing a moderately low percentage of fat could have their reproduction capacity less affected than animals on vitamin E



deficient rations with a high percentage of fat. GOTTLIEB and coworkers (1943) found that a diet low in fat produced very good results for the bioassay of vitamin E.

Comparing in table 3 groups 4 (+ E + cod liver oil) and 6 (+ E + lard), it is clear that the group which was fed cod liver oil exhibited a reproduction performance significantly lower than that of the animals which were given lard. Thus, even when high amounts of tocopherol are given to rats, qualitative differences in dietary fats may produce significant difference in the reproduction record of the animals. It may be that the highly unsaturated fatty acids of, for instance, cod liver oil greatly increase the requirements of vitamin E as compared with the unsaturated fatty acids of some other fat, lard, for example.

### Summary.

The influence of two different fats, viz., cod liver oil and lard, on the reproduction capacity of vitamin E deficient rats has been studied. Six groups of rats, kept for 10 weeks since weaning on the experimental rations prior to mating, were fed six purified diets, the differences among them being as follows: group 1, — E — fat; group 2, + E — fat; group 3, — E + 20 % cod liver oil; group 4, + E + 20 % cod liver oil; group 5, — E + 20 % lard, and group 6, + E + 20 % lard.

Groups 1 and 2 exhibited the poorest reproduction records, probably as a complication of the rigid exclusion of dietary fat, which produced in these animals a lower caloric intake and a deficiency of essential fatty acids.

The reproduction performance of the animals which were fed the vitamin E deficient diet containing cod liver oil (group 3) was significantly poorer than that of the animals which were fed lard (group 5). This shows once more that qualitative differences in dietary fats may greatly influence the sterility symptom in vitamin E deficient rats.

Furthermore, the reproduction record of the animals fed high amounts of vitamin E (20 mg per cent of tocopherol acetate) plus cod liver oil (group 4) was affected to a larger extent than that of rats fed lard (group 6). This shows that two different fats may influence to varying degrees the requirement of vitamin E for reproduction.

A correlation of the changes found in the adipose tissue with the reproduction performance of the various groups, points to a causal relation common for both symptoms (peroxidation and coloration of adipose tissue, and sterility): peroxidation of certain highly unsaturated fatty acids in the absence of the antioxidant effect of vitamin E.

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## Permeability of the Amphibian Skin.

### II. Effect of Moulting of the Skin of Anurans on the Permeability to Water and Electrolytes.

By

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In the course of investigations into the osmotic regulation in amphibians the permeability of the skin to water and salts was sometimes found to show unexpected fluctuations, even under well defined experimental conditions. These fluctuations could be demonstrated as being connected with the moulting processes. They have been studied more closely on *Bufo bufo* (L.), *Rana temporaria* L. and *R. esculenta* L.

In amphibians the outer cornified layer of the epidermis will separate at intervals from the underlaying tissue and then be shed almost in one piece. In toads and frogs, bursting of the old epithelium has been observed to occur along the midline of the ventral side of the animals. They very often eat the skin after shedding. In such cases the animals start eating the skin ends separating from the lower lips, whereafter the rest may be drawn into the mouth so completely that no remnants are left in the container. That moulting frogs may eat their shedded skin has previously been observed by *e. g.* FISCHER-SIGWART (1897), KNAUER (1879). FROST (1932), however, never found different species of frogs and toads eating the cast skin.

#### Technique.

The experiments were performed on starving animals adapted to tap water. They were kept in circular steel wire cages fitting into

250 ml beakers. Water was added to cover the animals, however, leaving just sufficient space between the water surface and the lid of the cage to make air breathing possible.

It has often been demonstrated that the amphibian skin is permeable to water and to electrolytes. In water, a frog or a toad will absorb water and lose ions by diffusion through the skin. In the present experiments the water absorption was measured by weighing animal + cage at suitable intervals of, generally, about one hour. The cage was suspended on one arm of a balance after the adhering water had been removed by drying with filter paper.

The loss of ions to the surrounding distilled water was followed by means of electric conductivity measurements. A Philip's Philoscop (a. c., 1000 cycles) was used in connection with a pair of dipping electrodes which were placed directly in the vessel containing the experimental animal. In the following, the measured resistance directly represents concentrations, *i. e.* mmol/l of a solution of NaCl of the same conductivity at the same temperature. Net uptake or net loss of Na and Cl in dilute salt solutions was determined by chemical analysis of the water in which the animals were kept. Cl was determined according to REHBERG in the modification of SCHNOHR (1934). For Na, the method of HOFFMAN and OSGOD (1938) was used in the modification described by JØRGENSEN, LEVI and USSING (1946). Total influx of Na has been measured by means of radioactive  $\text{Na}^{24}$  as described previously (*loc. cit.*).

The above mentioned methods of measuring water and electrolyte movements across the skin are of course based on the supposition that the animals do not swallow water and/or void urine between subsequent weighings or conductivity measurements.

As regards the uptake of water through the mouth, it is generally agreed that amphibians do not drink in dilute solutions such as tap water. Also the present results are incompatible with any noticeable intake of fluid through the mouth. Such an intake would be easily detectable as irregularities of the slope of the curves representing weight increase in water without accompanying changes in electrical resistance of the water (*cf.* below). Weight increase in water therefore indicates that water has been absorbed through the skin. On the other hand, the measured increase in weight will only equal the total amount of fluid absorbed if no urine is lost simultaneously to the surrounding water. It may be seen from the weight curves that the amphibians used really do void discontinually. A typical example of such a curve is shown in fig. 1. The increase in conductivity of the water and the increase in weight of the animal, a toad, is quite smooth until a momentaneous increase in conductivity sets in simultaneously with a fall in weight of the toad, indicating voiding. In this experiment, the delivery of about 0.1 ml urine would easily have been detected as a hump of the weight curve and — especially — of the conductivity curve. Several experiments of this kind have been performed even with saltloaded animals where voiding of urine in quantities much smaller than 0.1 ml would have been observed without difficulty.

It was always found that animals, when kept undisturbed, stored the urine in the bladder and only voided at longer intervals. Especially in the case of *B. bufo*, long lasting constant increases in weight may be found which are interrupted by a sudden decrease in weight at the voidings. Animals have been observed which did not void voluntarily for more than 8 hours, even though weighings were performed about every half hour. Frogs usually empty their bladder more frequently than do toads, and very often in connection with the weighing pro-

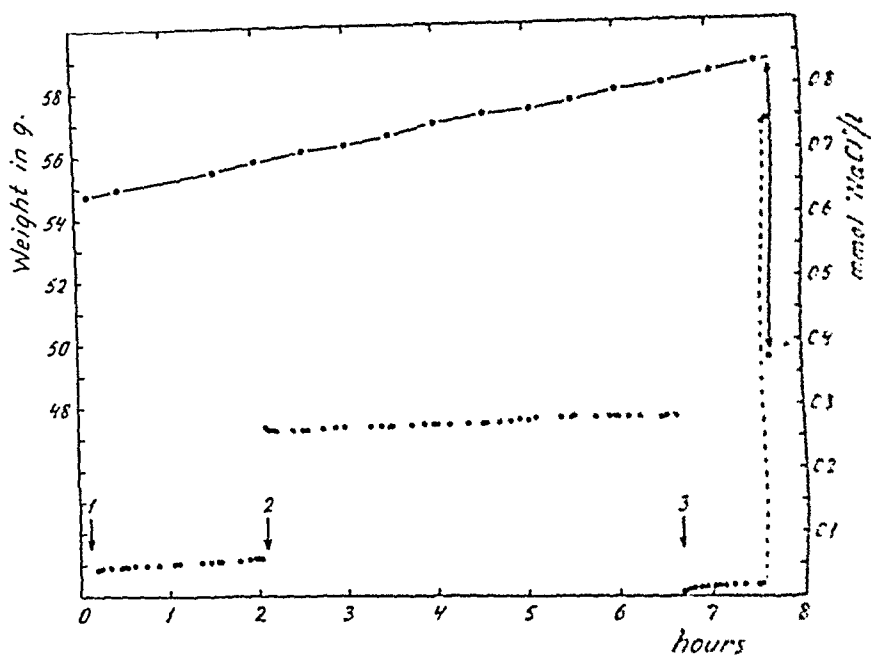


Fig. 1. At arrow 1 a toad (*Bufo bufo*) adopted to life in the cage was placed in 100 ml distilled water. At arrow 2 some NaCl solution was added. At arrow 3 changed to distilled water. Abscissae: time in hours. Ordinates: weight of the toad in g (left) and conductivity of the surrounding water (right). Conductivity expressed as equivalent concentration of NaCl.

cedures. It is well known that amphibians are inclined to deliver urine when disturbed in some way.

These observations on discontinuous voiding obviously do not exclude the possibility that smaller amounts of urine may constantly escape storage in the bladder and flow directly into the surrounding water. Normally, however, this seems not to be the case. When a frog or toad was placed in distilled water (without stirring) and the changes in resistance followed by placing the electrodes directly in the water in different positions relative to the animal, it was found that the increase in conductivity near the cloaca was not more rapid than near other parts of the animal, thus indicating that the measured increase in electrolyte content of the water is not due to a more or less continuous delivery of urine.

## Results.

### Water Permeability of the Skin during Moulting.

In the case of *Bufo bufo*, 14 single observations on water absorption during moulting were made, whereas water uptake has been followed only during 5 and 2 moulting periods, respectively, of *R. temporaria* and *R. esculenta*. The results were, however, the same in all cases. They show a strongly increased water influx through the skin in connection with shedding of the old epidermis. An example is given in fig. 2 depicting measurements

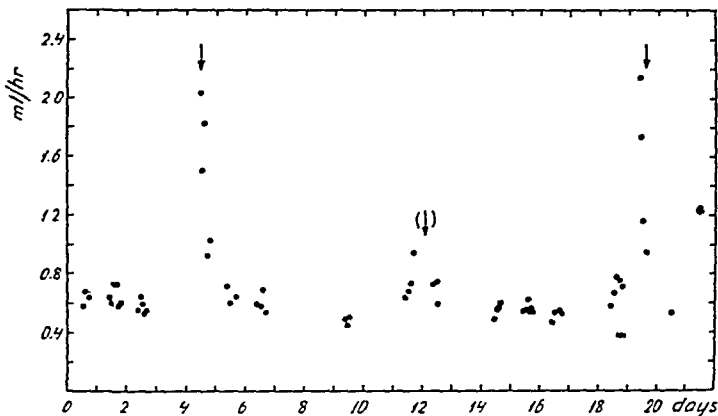


Fig. 2. Water absorption through the skin of a toad weighing about 37 g. Temp. 22–23° C. Ordinates: absorption in ml of water per hour. The arrows indicate moulting. Abscissae: time in days.

of water absorption of the same toad during a period of about 3 weeks. Mostly, several determinations of the rate of fluid uptake were performed daily. Throughout the experimental period 3 moults occurred. Two took place during daytime and both were followed by increases in water absorption up to 3–4 times the normal values. The third moult occurred during the night so that the actual uptake during moulting has not been determined. However, the value measured the day before was higher than normal. Fig. 3 is typical of the conditions in frogs, which, as already mentioned, behave very much like toads. Apparently, the period during which the moulting processes affect the water permeability of the skin may vary to some extent. Sometimes, a water uptake definitely above normal can be observed 12 hours before and after the shedding of the skin actually takes place. But often normal permeability is preserved until and is regained some few

hours before and after the observed moult. In each case the time during which excessive water permeability may be measured seems to be confined to the period when the old cornified stratum is separated from the underlying tissue.

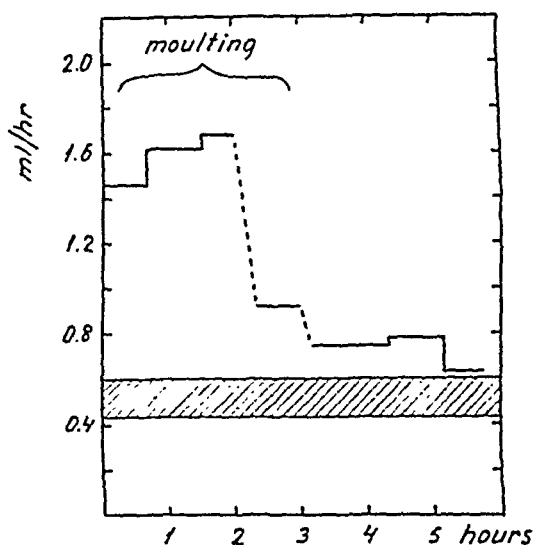


Fig. 3. Water absorption through the skin of a frog (*R. esculenta*) during mouling. Weight of the frog c. 46 g. Temp. 22—23° C. Ordinates: absorption in ml of water per hour. The hatched area indicates normal level of fluid absorption. Abscissae: time in hours.

### Loss of Electrolytes during Mouling.

The increase in water permeability of the skin during mouling was always accompanied by an increase in electrolyte permeability. A typical example is that of fig. 4. The loss of electrolytes was followed directly by determining the electric resistance in the surrounding water. Shortly before mouling the experimental animal, a toad, was placed in distilled water. At the instant indicated by the arrows the water was renewed. The hatched area in the lower part of the figure indicates the level of salt loss through the skin of the non-mouling animal. It is seen that the ion permeability of the skin was considerably higher than normal already at the start of the period of observation. The extreme values recorded during the periods designated by a, b, c, and d are most probably caused partly by urine voided during these periods. Disregarding these values, a pronounced peak in salt excretion through the skin is found coinciding with the separation of the

cornified epithelium. This separation occurred during the period indicated by the black area at the bottom of the figure. The simultaneous loss of ions through the skin amounted to about 20 times the normal values.

It is not known with certainty how long prior to and after the actual moult the permeability is increased. In one case normal permeability was found 3—4 hours after shedding of the skin. In other cases considerably longer periods were required. The

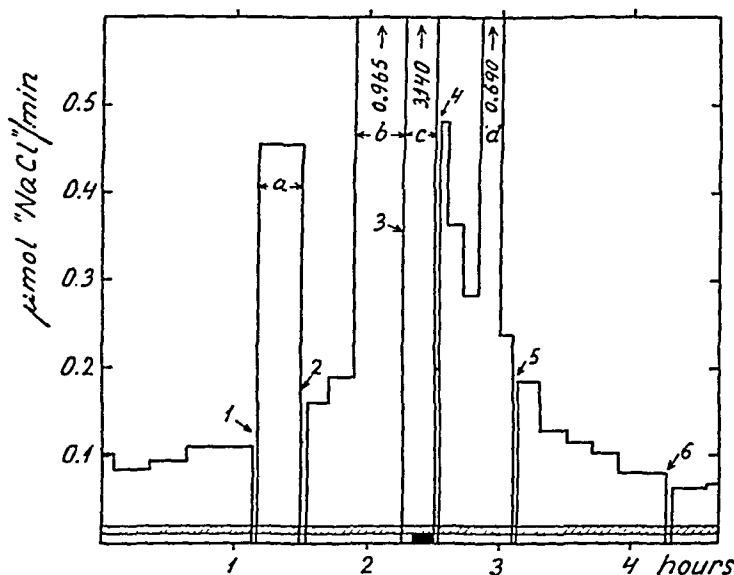


Fig. 4. Loss of ions through the skin of a toad during moulting. Weight of the toad c. 52 g. Temp. c. 23° C. Abscissae: time in hours. Ordinates: loss of  $\text{NaCl}$  in  $\mu\text{mol}$  per minute. Further explanation in text.

same holds for the time from the beginning increase in permeability until the skin was cast. Probably, the changes in electrolyte permeability and the changes in water permeability are parallel.

#### Na Influx through the Skin during Moulting.

It was shown above that the electrolyte permeability of the skin is increased during moulting. This was true for all species investigated: *B. bufo*, *R. temporaria* and *R. esculenta*. As the greater part of the electrolytes lost through the skin presumably consist of Na and Cl, an increased loss of NaCl during moulting should be detectable also when the experimental animals are kept in dilute salt solutions, for example tap water. In several cases, the net changes in the Na and Cl contents of tap water



Table.

The frogs were kept in about 3 mmol/l NaCl, the toads in 1–5 mmol/l NaCl. The surface areas (s) were calculated from the weight (w) according to REX (1937) using the formula  $s = 9.0 \cdot \sqrt[3]{w^2}$  for *R. temporaria* and  $s = 6.0 \cdot \sqrt[3]{w^2}$  for *B. bufo* Temp. 22–23° C.

Rana tem- poraria no.	weight in g	sur- face in cm <sup>2</sup>	non-moulting animals			moulting animals		
			equiv. Na/hr., 100 cm <sup>2</sup>					
			influx	net	outflux + loss through kidneys	influx	net	outflux + loss through kidneys
10 .....	24	75	5.8	± 0	5.8	12.7	— 4.1	16.8
11 .....	22	71	4.8	— 3.4	8.2	13.1	+ 0.6	12.5
43 .....	30	87	6.8	— 0.7	7.5	9.6	— 1.8	11.4
			6.6	+ 0.6	6.0			
Bufo bufo no.								
37 .....	32	61	1.4	+ 0.9	0.5	3.4	— 2.6	6.0
			2.0	+ 0.5	1.5			
			1.1	— 1.1	2.2			
			2.2	— 1.6	3.8			
38 .....	31	60	1.5	± 0	1.5	4.7	— 2.4	7.1
			2.1	+ 0.3	1.8			
			1.8	± 0	1.8			
			2.1	— 1.0	3.1			
40 .....	35	64	2.8	+ 0.8	2.0	4.0	— 0.7	4.7

in which frogs or toads were living have been followed chemically over a period of several days. During some of the experimental periods moults have occurred, however, often without any pronounced net loss of Na and Cl. Since it is well-known that amphibians are able to take up salt through the skin from dilute salt solutions (see *e. g.* KROGH 1939) nothing can be concluded from measurements of net changes in salt content concerning the absolute amount of salt lost or gained. It is necessary to determine the total influx of salt through the skin and the total loss through skin and kidneys. Such determinations were carried out by means of the radioactive isotope Na<sup>24</sup>. The data obtained on animals studied during as well as before and after the moulting period are summarized in the table. In all cases, a pronounced increase in Na influx occurs simultaneously with the moult. The net changes, however, usually are less conspicuous, especially in the case of *R. temporaria*. The total excretion, viz.

outflux through the skin and loss through the kidneys, was therefore also increased during moulting in tap water.

The question arises whether the increased Na influx occurs simultaneously with the increased skin permeability or whether it is caused by the salt depletion following the loss of NaCl. It has been shown (JØRGENSEN 1948) that salt depletion actually may stimulate the influx of salt through the skin. During moulting, however, the increase in influx and outflux seems to be

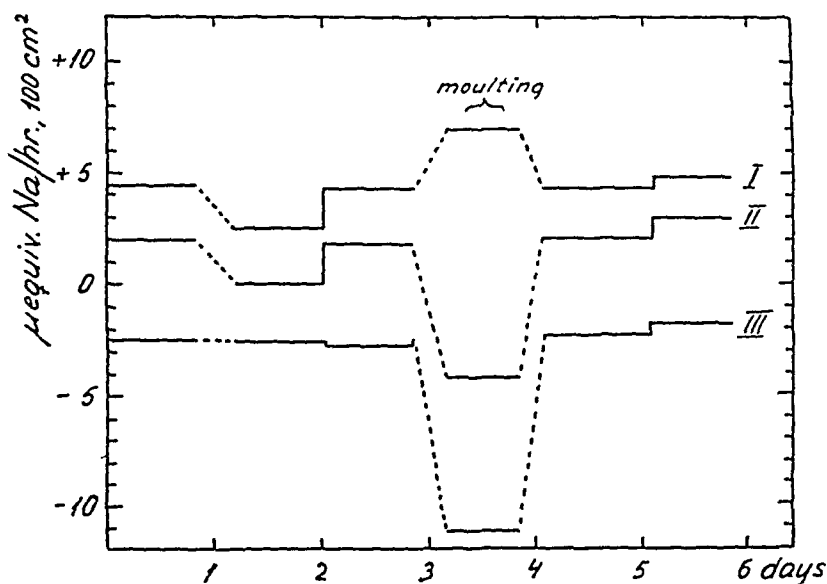


Fig. 5. Influence of moulting (toad) on I influx of Na through the skin, II net loss or uptake of Na, and III outflux through the skin + renal loss of Na. Ordinates:  $\mu\text{equiv. Na/hr. } 100 \text{ cm}^2$ . Abscissae: time in days (hours resp.).

simultaneous, so that the processes connected with the moulting seem to be responsible for both reactions. This may be supported by fig. 5 and especially fig. 6. In fig. 5, influx, net changes, and total excretion have been determined in several consecutive periods, during one of which moulting took place. The respective toad had lost salt previously during a prolonged stay in running distilled water; consequently, a net uptake of salt occurred, except on the day of moulting. The increased loss on that day apparently did not induce an increased influx of Na. Except for the period when the skin was shedded, the Na influx was not increased, although a net loss of Na persisted at the end of the period during which the moulting occurred. In fig. 6 an example is shown where the measuring period within which the skin was

shedded is rather short, viz. about 7 hours, compared with the moulting time. If the period of increased influx is lagging behind the period of increased outflux, the influx should be less pronounced, but should continue in the following measuring period when the direct effect of the processes leading to skin shedding and increased permeability have disappeared. This is obviously not the case. On the contrary, influx and outflux both are more pronounced than normally found during longer measuring periods, and the effects are restricted to the moulting period proper. It must therefore be concluded that increased influx as well as outflux of salt are simultaneous phenomena which coincide in time with the shedding of the skin. The net result of the two processes which, *i. a.*, necessarily depends on the salt concentration of the medium may be a real loss of salt such as has always been found in the toad. Another possibility is that the net changes

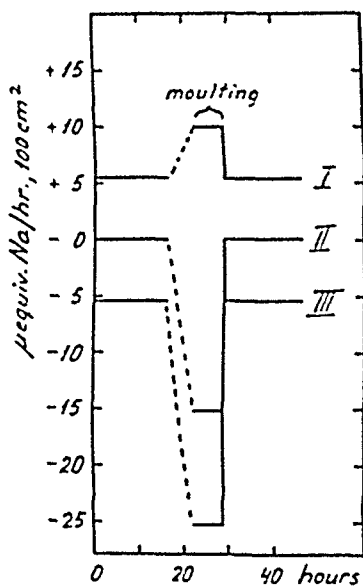


Fig. 6. For explanation see fig. 5.

cannot be distinguished from the net changes occurring in normal animals which do not moult, as frequently found in the case of *R. temporaria*. *R. esculenta* has not been investigated in this respect. However, the differences observed between *B. bufo* and *R. temporaria* may be quite incidental. The material available is too small to allow definite statements concerning possible species — specificities or ecological consequences.

### Summary and Comments.

It has been shown that during moulting of *Bufo bufo*, *Rana temporaria*, and *Rana esculenta*, the permeability of the skin to water and salt is considerably increased as compared with the permeability prevailing in non-moulting animals. The increase in water permeability amounts to 3—4 times normal and that in salt permeability to about 20 times the normal values. Maximum permeability coincides with the separation of the cornified epi-

thelial layer from the underlying tissue. The mechanisms involved in the change of skin permeability accompanying the moult are not known.

Simultaneously with the increased permeability, the active absorption of salt from dilute solutions (about 1—3 mmol NaCl/l) was found to be enhanced. This fact has a certain resemblance to the conditions prevailing after increased skin permeability produced by adrenaline (JØRGENSEN 1947). Here, too, an accompanying increase in influx of NaCl could be demonstrated (USSING 1949).

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## The Response of the Frog's Taste Fibres to the Application of Pure Water.

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The glossopharyngeal nerve of the frog offers excellent conditions for studying the sensitivity of the tongue to different kinds of stimuli. PUMPHREY 1935 was the first who recorded action potentials from this nerve applying different chemical stimuli on the frog's tongue. He found that the tongue possessed endorgans responding to salt and acid but not to sweet or to bitter substances. He also observed that strong chemical stimuli produced an activity in thinner fibres which he looked upon as pain fibres.

In the cat ZOTTERMAN 1935 showed that the fibres of the glossopharyngeal and lingual nerve which responded to taste stimuli were of a smaller diameter than those responding to tactile stimulation. It was also found that the cat lacked endorgans responding to sweet tasting solutions, an observation which was confirmed by PFAFFMANN 1942.

When listening-in to the impulse traffic in the glossopharyngeal nerve of the common Swedish frog (*Rana temporaria*) it was observed that the application of common tap water or distilled water upon the tongue elicited an immediate and massive volley of impulses in the larger fibres of the glossopharyngeal nerve. The present investigation was undertaken in attempt to analyze this rather unexpected phenomenon further.

## Technique and Procedure.

The common Swedish frog (*Rana temporaria*) was used in this investigation which was started in November 1948. Thus all experiments were performed on winter frogs, which were decerebrated and pithed. The glossopharyngeal nerve was dissected out from its most central end and the frog was placed on its back in the moist chamber. By means of a thread sewn into the mandible this part was lifted up thus exposing the upper surface of the tongue. The action potentials were recorded by means of an ordinary resistance capacity coupled amplifier, previously described (ZOTTERMAN 1936) and a cathode-ray tube.

The test solutions were applied in different ways, by stroking the tongue with cotton wool soaked in the solutions or by pouring the fluid from a pipette. The best way of applying the stimulus was however by means of an (insulated) burette arrangement from which a fine stream of the test solution was directed upon the tongue, thus keeping the mechanical stimulation of the different solutions constant. The tongue was washed by Ringer solution between every test.

## Results.

*The water effect.* When the tongue was imbedded in the mucin-rich saliva the activity of the glossopharyngeal nerve is of very low intensity. Only one drop of tap or distilled water is however enough to elicit a very high activity in the large nerve fibres and when a few ml of water is poured upon the tongue there follows a massive discharge of impulses in the large fibres (see Fig. 1 A). The discharge increases rapidly and goes on for a minute or more with slowly decreasing frequency.

This water discharge was very quickly abolished by pouring Ringer solution upon the tongue (see Fig. 2). Ringer solution itself produced generally a rather small response (see Fig. 1 B). When water is applied the first impulses are produced by the deformation of tongue's surface brought about when the water hits the tongue. By comparing the records Fig. 1 A and Fig. 1 B it will be seen that the specific effect produced by water starts very quickly within a tenth of a second after the impact of the water upon the tongue. The abolishing effect of Ringer solution starts also promptly but not as rapidly as the water effect. Generally a very marked reduction in frequency is observed within 0.2 sec. after the application of the Ringer solution. (Fig. 2.)

NaCl of a concentration about 0.6 % acted very much like Ringer while increasing concentrations of NaCl (1—3 %) elicited

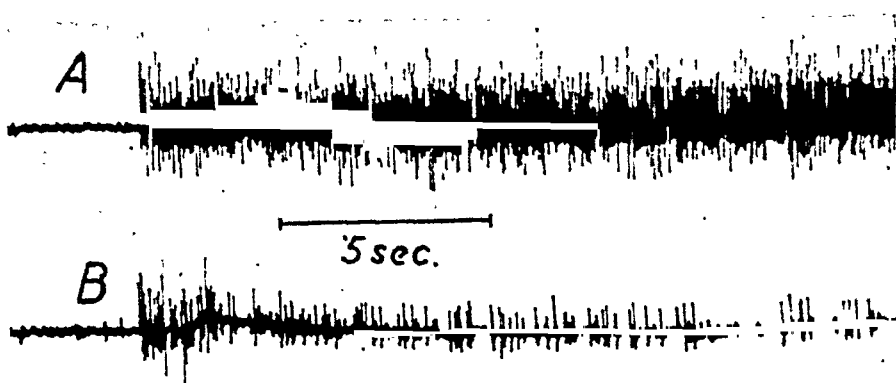


Fig. 1. Leads on the glossopharyngeal nerve of the frog (*R. temp.*).

- A. response to 2 ml of tap water poured upon the tongue from a burette;  
B. the response to the same amount of Ringer solution.

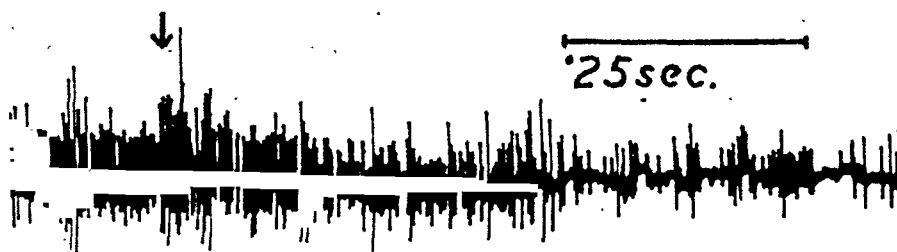


Fig. 2. The water effect was started 20 sec. earlier. At the arrow 2 ml of Ringer solution is squirted upon the tongue.

correspondingly stronger discharge of action potentials apparently from the same kind of fibres, which respond to water. When testing gradually diluted NaCl solution from 0.6 % downwards it was found that the response in many cases increased when the concentration fell below 0.1 %.

### **The Effect of Bitter Tasting Substances.**

The generally used bitter tasting substances strychnine nitrate and quinine sulphate have been tried on this frog preparation. In order to avoid the water response the test substances must be administered in Ringer solution. It was thus found that these substances did not set up any impulses in the large fibres responding to water, touch, salt or acid solutions. Careful examination of the records showed however that there appeared small and slowly conducted spikes. This activity of the finer nerve fibres was fairly weak when applying strychnine in concentrations 0.01 % to 0.1 % which are very bitter to our taste. In order to test a more natural food for the frog, a frog's saliva extract of flies was made. When these extracts were applied to the tongue a definite response from the small fibres was observed (Fig. 3 B). Distilled water extracts on flies (Fig. 3 C) also produced a fine fibre response besides an effect on the large fibres.

Small fibre response is seen in addition to the large spikes when applying strong mechanical stimuli, strong NaCl solution and strong acid solutions (pH below 3). There is therefore no doubt that noxious stimuli set up impulses in fine fibres. There is however no apparent reason to assume that saliva- or water-extracts of flies should stimulate specific pain fibres. It seems to me more likely that the small spike response to these fly extracts is due to thin fibres, the endings of which are specifically sensitive to certain substances of the extracts as well as to bitter tasting substances. Thus the frog's tongue could be assumed to possess specific receptors subserving a chemical sensitivity different from that subserved by the large afferent nerve fibres.

Quinine in a concentration of 0.3 % in Ringer solution had two distinct effects. Upon application the solution gave rise to a massive volley of slow spikes which summated up in a characteristic way in waves, displaying a very high activity of the small afferent fibres. The response lasted for many minutes (see Fig. 4).



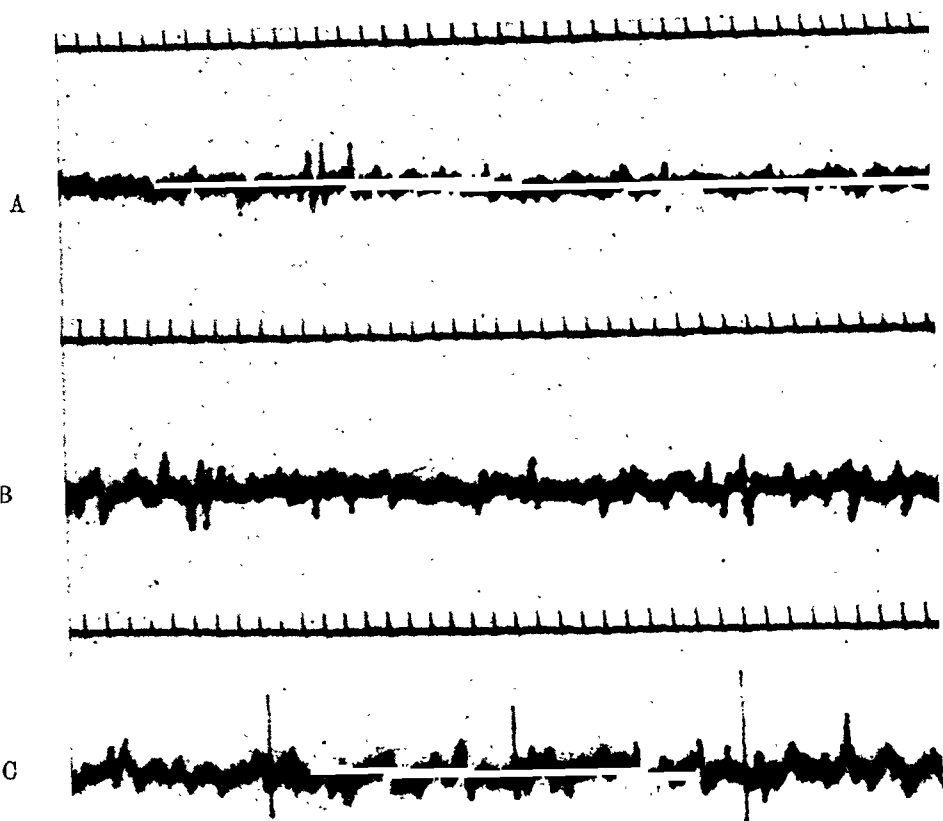


Fig. 3. Action potentials from the frog's glossopharyngeal nerve.

- A. control;  
B. after exposing the tongue to an extract of flies made with frog's saliva and  
C. to an extract of flies made with distilled water. The records made about 2 minutes after the application of the extracts. Time 50 cps.

But later, after the application of such strong quinine solutions on the tongue it was found, that the water effect could not be produced any longer (Fig. 4 C). Further the response to mechanical stimuli was abolished or very much reduced. This effect on the large afferent fibres persisted for hours.

Thus strong quinine solutions in Ringer stimulated very highly the small afferent fibres while it produced a lasting paralysis of the large fibre endings responding to water as well as to tactile stimuli. The interesting fact was that the small fibre activity could be reduced and abolished by washing the tongue with Ringer but the excitability of the large fibre endings did not return. Thus the large fibre endings are definitely knocked out, leaving the

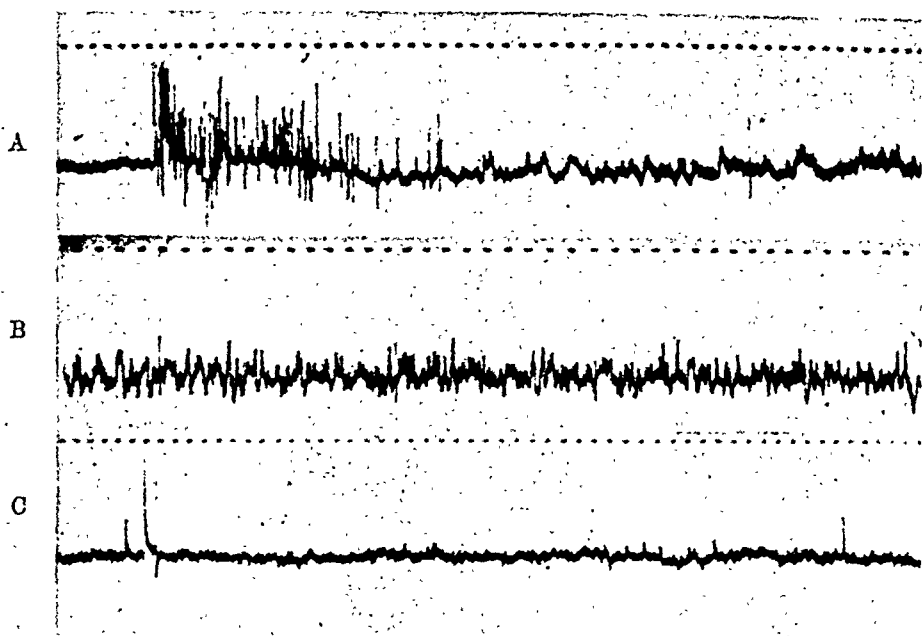


Fig. 4. Records from the frog's glossopharyngeal nerve showing the effect of quinine sulphate, 0.3 % in Ringer solution.

- A. 2 ml of quinine solution are poured upon the tongue;
- B. another preparation showing the lasting effect of quinine upon the small fibre endings;
- C. 2 ml of tap water does not produce any effect upon the tongue which has previously been exposed to quinine. Time 25 cps.

signalling entirely to the small fibres. This looks very much like what one would expect from an *anestheticum dolorosum*, and it seems very likely that quinine at this high concentration exerts a noxious action giving rise to nociceptive reactions. This action is however not produced by strychnine nitrate. Even as strong a strychnine solution as 1 % in Ringer has no effect whatever on the large fibre endings of the frog. That quinine produces local irritation and anesthesia has long been known. It has also been used clinically as a local-anesthetic but has been abandoned as the anesthesia often persisted for several days, which is due to necrosis of the axones with subsequent regeneration (SALM 1940). When the saphenous nerve of the dog was soaked in Ringer solution containing 0.3 % quinine sulphate for c:a 15 minutes, the  $\delta$ -elevation however disappeared entirely, while the  $\beta$ -elevation still remained although somewhat reduced.

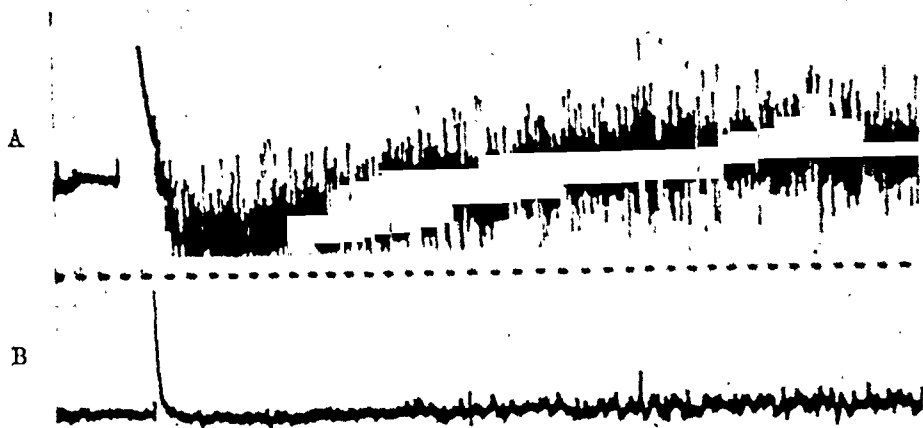


Fig. 5. Records from the frog's glossopharyngeal nerve showing  
A. the response to isotonic  $\text{CaCl}_2$  solution;  
B. the same after previous exposure to quinine sulphate which paralyzed the large fibre endings. Time 25 cps.

### The Effect of Different Ions on the Water Effect.

The response produced by Ringer was not changed by adding 5.5 % cane sugar to the solution. But this concentration of sugar in water produced an effect indistinguishable from that of water alone. This proved that the frog's tongue has no nerve endings responding specifically to sugar and that the water effect is not due to osmosis, but must be due to the withdrawal of ions from the surface of the tongue. This assumption was strengthened by recording the effect of a series of isotonic solutions of different salts  $\text{KCl}$ ,  $\text{KBr}$ ,  $\text{KJ}$ ,  $\text{NaBr}$ ,  $\text{NaJ}$ , etc. All these salts diminished the water effect although to different degrees. The strongest inhibitory effect was produced by  $\text{KCl}$ . After having exposed the tongue to isotonic  $\text{KCl}$ , the spontaneous impulse traffic in the nerve was entirely abolished and the water effect was thereafter temporarily reduced and so was the effect of mechanical stimuli.

An opposite effect is exerted by  $\text{CaCl}_2$ . An isotonic solution of this salt produces a massive volley of impulses (see Fig. 5). There is however an important difference between this response and that produced by water, for  $\text{CaCl}_2$  excites also the small fibres. This stimulating effect upon the small fibre endings can best be observed after having exposed the tongue to 0.3 % quinine sulphate

solution since this paralyzes the large fibre endings but leaves in action the endings of the small fibres. We see in Fig. 5 B that the large fibre response is abolished but there is a very distinct response of the small fibres to  $\text{CaCl}_2$ . In Fig. 4 C it is equally well seen that under this condition water does not produce any effect upon the small fibre endings. Thus the water effect is an affair entirely of the large fibre endings.

### Discussion.

The findings speak very much in favour of the view that the water effect is produced by removing free ions from the surface of tongue. It is further evident that it is the endings of the larger nerve fibres which display a specific sensitivity in this respect. This water effect has been elicited from the tongue of *Rana temporaria* during the winter season and lately also on a few specimens of Hungarian winter frogs (*Rana esculenta*). It was first observed in November in frogs which had been caught after having reached their winter hiding place below the lake water surface. It does not occur in the tongue of the cat and we do not know as yet whether it is present in the summer frog.

It would be interesting to know the physiological significance of this water effect. The fact that the response is limited to the large nerve fibres implies that it does not produce any nociceptive reactions. It is tempting to suppose however that the water effect may reflexly contribute in keeping the mouth of the frog closed as well as to inhibit the respiratory movements when under water. The effect has so far only been obtained from the glossopharyngeal nerve and control experiment on dorsal skin nerves did not show any effect of this kind. Thus it looks as though the effect might inhibit the respiratory movements and protect the airways and the lungs from being filled with water analogous to the reflex inhibition in mammals.

The phenomenon might however also subserve another biological purpose. The amphibians who live in fresh water very carefully retain their mineral salt by reabsorption in the renal tubuli and excrete a very hypotonic urine. It does not seem unlikely that the water on the tongue reflexly keeps the mouth closed, thus reducing an otherwise obvious increase of the intake of water and a subsequent greater loss of salts due to an increased diuresis. The specific

sensitivity of these nerve endings towards fresh water should thus take a part in the regulatory mechanisms for stabilizing the ionic balance.

### Summary.

1. Fresh water applied to the frog's tongue produces a massive volley of large fibre impulses in the glossopharyngeal nerve lasting for some minutes. (The observations were made upon winter frogs.)

2. This water response of the large fibre endings is abolished by Ringer solution, NaCl and some other salts in isotonic solutions but not by isotonic cane sugar solution.

3. KCl exerts a general inhibitory effect upon the large fibre endings of the tongue, while  $\text{CaCl}_2$  does not only fail in abolishing the water effect but excites also the small fibre endings of the tongue.

4. Quinine sulphate, 0.3 % in Ringer, stimulates highly the endings of the small fibres and produces a long lasting paralysis of the large fibre endings.

5. The physiological significance of the water effect upon the frog's tongue is briefly discussed. It is suggested that it takes part in the regulatory mechanisms of the ionic balance.

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## The Influence of Skin Temperature on the Number of Reacting Cold Spots.

By

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BLIX (1883) and GOLDSCHIEDER (1884) by means of suitable stimuli demonstrated that sensation of cold in the skin is released from isolated points. Later it has been shown that the localization of these sensitive spots in the skin changes from one examination to another (GOLDSCHIEDER 1898, TROTTER and DAVIES 1909, BORING 1916, WATERSTON 1923), and that moreover the number of spots found within a given area is subject to change (GOLDSCHIEDER 1898, WATERSTON 1923), so that in order to discover all the spots which can react to cold it is necessary to make several examinations of the same area (GOLDSCHIEDER 1898, WATERSTON 1923, DALLENBACH 1927). If this is done it is found that the localization of the spots from which a cold reaction can be released is very constant (DALLENBACH 1927). An opinion not in conformity with the above mentioned is advanced by JENKINS (1941), who from results obtained by serial tests of skin areas including the differentiation of the strength of the induced cold reaction concludes that the sensation of cold is not excited from points but from larger areas of the skin surrounded by refractory zones. The strength of the reaction depends upon the localization of the stimulus within the area.

The anatomical basis of the induced cold reaction has not been clarified. The discovery of KRAUSE's end bulbs in the conjunctiva and in the skin close to a previously localized cold spot has caused these elements to be regarded as specific receptors of cold (STRUG-

HOLD and KARBE 1925, WEDELL 1941), although it is by no means always possible to find end bulbs in skin with distinct cold spots (DALLENBACH 1927, WEDELL 1941). (NAFE and WAGONER 1936) assumed that thermo-sensitivity depends upon the initiation of afferent impulses, which are released by means of the constriction and dilatation of the arterioles of the skin, whereas JENKINS (1941) considers that the probable receptors are free nerve endings assembled in groups of different densities, a strong reaction being released when many receptors are stimulated and a weak reaction when the receptors are few.

As stated above, the number and distribution of the cold spots vary considerably in the same skin area from one examination to another, a phenomenon which perhaps is connected with an alternating function between the individual receptors (WATERSTON). In the present work this variation in the number and localization of functioning spots has been examined at different skin temperatures by means of serial tests on a single area of the skin. The reaction of the individual cold spot to local temperature variations has also been examined in order to elucidate what factors govern the variations in the cold sensitivity of the skin.

### Technique.

The examinations were performed in a room in which the temperature could be maintained at a constant level within  $1^{\circ}$  C and where noise and other disturbances were avoided, so that the subject could concentrate upon the test.

The number of cold spots and their distribution in the skin were registered in the following manner:

By means of a rubber stamp an area of  $6\text{ cm}^2$  divided into 6 squares of  $1\text{ cm}^2$  was marked off on the skin of the volar aspect of the forearm, in a position so that no big vein stems ran below the skin, thus avoiding this source of local temperature variation. For some weeks the area was gone over several times for spots from which a cold sensation could be released, the testing device consisting of a brass container with a conical point, the end of which had a flat surface measuring  $1\text{ mm}^2$ . The container held water, the temperature of which was varied between  $10^{\circ}$  and  $50^{\circ}$  C. The thermal conductivity of the metal was considered to be sufficient to allow the temperature of the point to remain constant while the entire skin area was examined once. Cooling was performed by allowing the weight of the container (45 g) to press the point against the skin for about one second. It was then raised above the skin and not placed on the next small skin area until the subject had reported reaction or no reaction. The strength of the induced cold

reaction was not differentiated, this being too uncertain. Prior to the test the skin was uncovered for ten minutes, and before and after the examination the skin temperature was measured by means of a thermocouple and a light-spot galvanometer as previously described (BING and SKOUBY 1947). The skin temperature was recorded with an accuracy of  $0.5^{\circ}\text{C}$ , as variations in the manner of application and localization within the area tested caused fluctuations of this size. An ink mark was made on spots where a cold sensation was elicited, and after the examination these points were transferred to cellophane paper having a printed pattern corresponding to the pattern stamped on the skin. The ink marks on the skin were then removed. When several tests were made on the same day, an interval of at least 15 minutes was allowed between two tests on the same small area.

When testing the reactions from a single cold spot the forearm was kept immobile and the thermode was placed in an adaptable stand adjusted so that the point of the container just touched the skin spot to be tested. The first cold spot found during an examination was employed for the experiment. Then the cold thermode was flushed with water of  $30\text{--}40^{\circ}\text{C}$  for one to ten minutes, whereafter its temperature was lowered to  $10^{\circ}\text{C}$  by passing cold water through it. This change in temperature could be obtained in about 10 seconds. The cold spot was warmed and cooled 3—4 times only in a single experimental series in order to prevent fatigue to interfere with the results obtained.

## Results.

Skin areas in two subjects were examined for the purpose of ascertaining the number of cold spots at different skin temperatures and three test series were performed comprising 34, 33 and 13 examinations of a single skin area ( $6\text{ cm}^2$ ) for cold spots at different skin temperatures. In one person the number found increased from 17 to 93 spots with increasing temperature from  $25^{\circ}$  to  $33^{\circ}\text{C}$ , and in the other from 13 to 93 spots accompanying an increase in skin temperature from  $27.5^{\circ}$  to  $34.5^{\circ}\text{C}$ . Fig. 1 shows the number of reacting spots as a function of the skin temperature. The single experiments of this series were performed on 17 different days spread over one month. The curves show that the number of cold spots increases more than proportional to the increase in the skin temperature. It might be assumed that this is due to the increase in the difference between the temperature of the skin and that of the cold element. This possibility is examined by determining the number of cold spots in three test series, in which the skin temperature was the same during the tests, whereas the temperature of the cold element was varied from one test to another within a range of  $16^{\circ}$  to  $35^{\circ}\text{C}$ . The re-





sults can be seen from fig. 2, which shows that with rising temperature in the thermode the same number of cold spots is found until in one series a value lying  $3.5^{\circ}\text{C}$  below the skin temperature is reached, and in the other a value of  $2^{\circ}\text{C}$  below. Thereafter the number of reacting spots falls abruptly to 0 in agreement with earlier investigations (LEEGAARD 1891). The reason why warm skin manifests a cold reaction from more places than cold skin may perhaps be that the spreading of the stimulus is promoted by improved skin circulation. The effect of capillary dilatation is examined by inducing a slight ultraviolet erythema on a part of the skin area already tested several times and then in the course of five tests counting the number of cold spots in the exposed and in the non-exposed areas. No definite difference in the skin temperature in the two areas with different blood supply was observed, and no difference in the number of cold spots found. Therefore the higher number of spots in warm skin cannot be ascribed to stimulus diffusion caused by capillary dilatation.

The suggestion has been made (WATERSTON 1923) that the variation in the localization of the cold spots in a skin area is due to a constant alternation in the activity between a certain number of spots from which cold sensation can be released. It is possible that with a rising skin temperature there is an increased activity of the elements from which cold sensation can be released, when the skin is cold, or that the increase is caused by the activation of elements which never react when the skin temperature is low. This question is investigated by plotting in a common system of coordinates the localization of the reacting spots from 8 tests of a skin area of  $2\text{ cm}^2$  at a skin temperature of  $27.5\text{--}29.5^{\circ}\text{C}$  and also plotting the localization of the reacting spots in a single test in which the temperature of the skin in the area explored was  $34^{\circ}\text{C}$ . In the experiments performed at a skin temperature between  $27.5\text{--}29.5^{\circ}\text{C}$  the cold spots found varied between 5 and 10, the lowest values being found at the lowest skin temperatures. The total of spots from which cold sensation was released made 22. This indicates an alternation between different cold receptors, which is in agreement with findings from the investigation of other receptors *e. g.* light perceiving elements of the retina (BERGER and BUCHTHAL 1938). At a skin temperature of  $34^{\circ}\text{C}$  cold sensation in the single experiment performed was released from 34 different points, 20 of these corresponding to the cold spots found at the lower tem-

esses following a previous reaction or improves the conduction of impulses via afferent nerve fibres. Diffusion of the stimulus over a larger area cannot explain the phenomenon, as in warm skin it is possible to demonstrate cold spots which cannot be induced to react when the skin is cold, even when the stimulus is placed as close to the spots as possible.

### Summary.

The number of reacting cold spots within a given area of the skin increases with the skin temperature. In one series of experiments the number found increased from 17—93 spots with a rise in temperature of the skin from 25 to 33° C. This is due both to increased excitability of spots from which a reaction can be elicited at lower temperatures and to a reaction from spots in areas from which it is possible to induce reaction at high temperatures only.

Heating of the single cold spot to 30°—40° C does not affect its excitability. The increased excitability requires a change in the temperature in larger areas of the skin or the underlying tissues. The mechanism of the change in the mode of reaction is discussed.

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## A Method for the Determination of Blood Pressure in Intact Dogs.

By

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When performing experimental studies on hypertension produced in some way or other in dogs one needs a simple method for numerous daily estimations of the blood pressure continued during weeks or months. The method described by HAMILTON, BREWER and BROTMAN (1934) includes subcutaneous arterial puncture and gives accurate pressures but it is certainly only in the hands of an expert that it permits fairly frequent repetition without causing too much disturbance of the animal. The well-known VAN LEERSUM carotid loop method demands a preceding operation on the animals which is not always easy to perform successfully. Aside from this latter method other indirect methods of blood pressure estimations in intact animals have been described by amongst others ALLEN (1941) and RULE (1944), both using the ordinary auscultatory method including the compression of an artery by means of a cuff. In some preliminary experiments we have used a similar method but could not obtain reliable results.

BONSMANN (1934) described a method of estimating the blood pressure in the intact rat. He applied a cuff round the proximal part of the rat's tail and placed the distal part of the tail between a lamp and a photoelectric cell. This photoelectric cell was connected with an amplifier and an amperemeter was used as measure instrument. When the tail began to receive blood as the pressure in the cuff was successively decreased, the amperemeter

started to give a continuous deflection. A similar method was described by KERSTEN et al. (1947) using a cuff round one of the legs of a rat and applying the photoelectric cell on the paw.

The method described in the present investigation has been elaborated in order to give a fairly simple and exact method. The principle of the method adheres to the above-mentioned method (KERSTEN et al.) in that it includes the use of a cuff round one of the legs of the animal and a photoelectric cell with its light source applied on an interdigitale fold of the skin. It differs from KERSTEN's method in that it includes the use of a mirror galvanometer whereby the beginning of the pulsations in the distal part of the leg can be observed. This renders the reading off more exact as it is easier to observe the beginning of the pulsations than to make an exact observation of the beginning of a continuous deflection.

### Recording Device.

The photocell (alkaline) and the lamp were mounted on a clamp which could be applied on an interdigitale skin fold on the fore-leg and could be firmly attached by means of a screw adjuster (Fig. 1, A). The cuff was of the type generally used for children and was applied round the upper part of the forearm. The animal was lying on its side and the paw was fixed between sand-bags (Fig. 2) which was sufficient to prevent disturbing movements derived from breathing, heart-beats etc. It was essential to place the animal in a warm environment so as to prevent chills which will cause disturbing movements. The animals were soon accustomed to the arrangement and the use of sedatives was thus rendered unnecessary.

The photoelectric cell was connected with an amplifier (Fig. 3) giving impulses to a mirror galvanometer. The light beam from this galvanometer was thrown upon a focussing-screen. At the side of this screen a mercury manometer was attached (Fig. 4).

Fig. 3. Photocell — amplifier.

$R_1$ : 4 M  $\Omega$   
 $R_2$ ,  $R_3$ : 3 M  $\Omega$   
 $R_4$ ,  $R_5$ : 1.5 K  $\Omega$   
 $R_7$ ,  $R_8$ : 0.1 M  $\Omega$   
 $R_6$ : 3 K  $\Omega$   
 $R_{10}$ : 10 K  $\Omega$

$C_1$ ,  $C_6$ ,  $C_7$ : 1  $\mu$  F  
 $C_2$ : 0.01  $\mu$  F  
 $C_3$ ,  $C_4$ ,  $C_5$ : 25  $\mu$  F el. lyt.  
 $C_8$ : 16  $\mu$  F

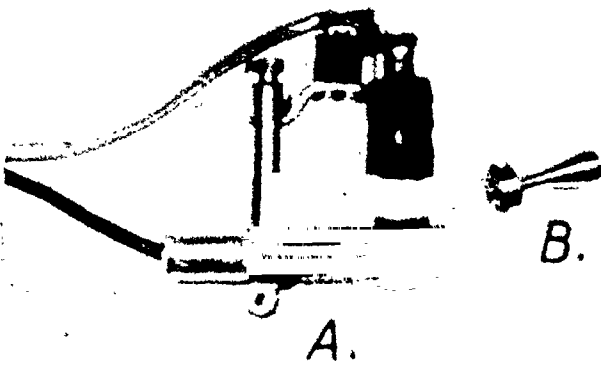


Fig. 1.

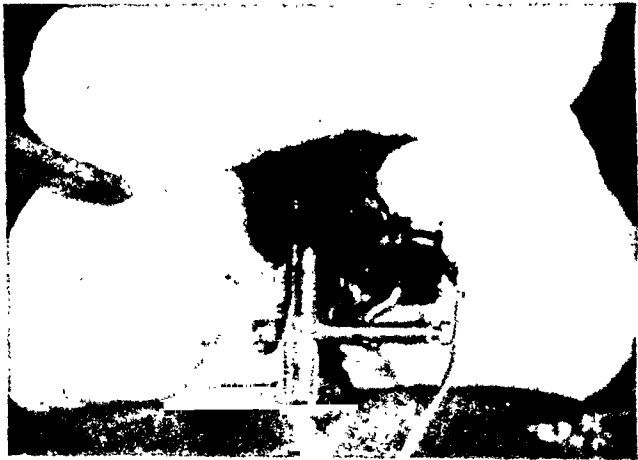


Fig. 2.

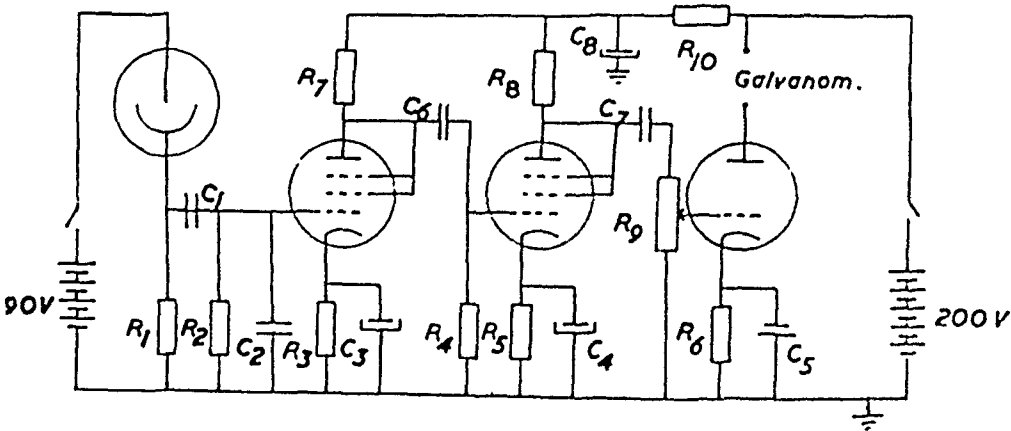


Fig. 3.

The estimation of the blood pressure is performed in the following way. The pressure in the cuff is raised to well above the presumable arterial pressure. On the screen the light beam is now immobile. The pressure is then slowly decreased (which can be

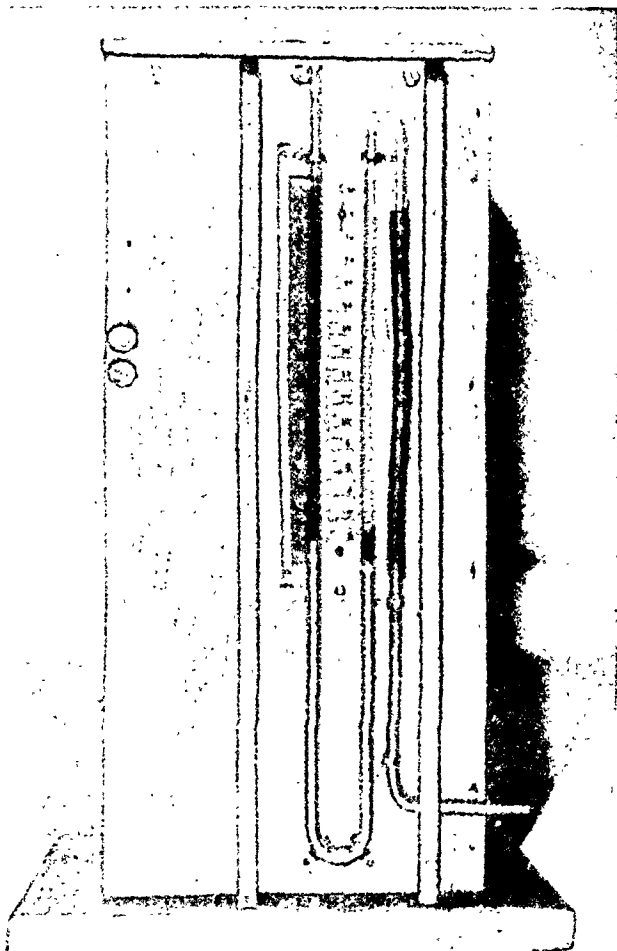


Fig. 4.

followed on the mercury manometer) and suddenly the light beam begins to show pulsations. At this moment the pressure is read on the mercury manometer. In order to get a vasodilatation in the interdigitale skin fold a 1 ‰ solution of histamine dihydrochloride is pricked into the skin by means of a small apparatus constructed like a bread-pricker (see Fig. 1, B).

It might be mentioned that the photoelectric cell can be con-

nected with a usual ECG-device with the addition of an H. T. battery in order to give the necessary voltage for the photocell (Fig. 5). The slide-vane of the ECG-apparatus is then used instead of the special focussing-screen mentioned above.

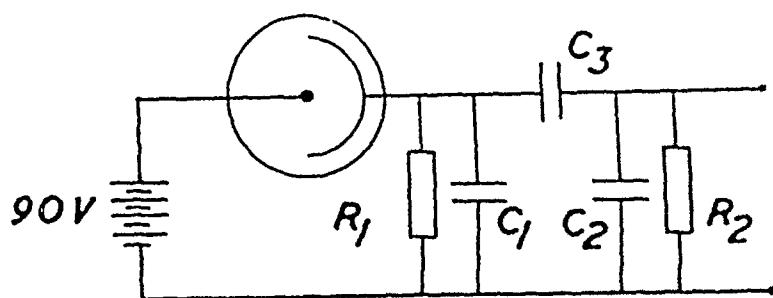


Fig. 5. Photocell — connection to ECG-device.

$R_1, R_2: 3 \text{ M } \Omega$   
 $C_1, C_2: 0.002 \text{ } \mu \text{ F}$   
 $C_3: 1 \text{ } \mu \text{ F}$

### Experimental.

In order to test the blood pressure recording apparatus, described above, the following experiments on anaesthetized dogs were performed.

Dogs were anaesthetized with nembutal (0.03 g/kg body weight) and the blood pressure was recorded from the radial artery by means of a cannula connected with a mercury manometer. On the contra-lateral fore-leg the blood pressure was measured with the apparatus described above. The readings were then compared with the blood pressure records on the kymograph. In order to obtain high blood pressure values adrenaline was given intravenously. Low blood pressure values were obtained through the intravenous injection of "Adynol" (Astra) — a preparation containing adenylic pyrophosphate. Thus the blood pressure readings covered a range from 75 to 260 mm Hg.

Fig. 6 gives the graphical representation of the correlation between the blood pressure values obtained with the two different methods. It must be pointed out that the blood pressure value obtained with the method described in this paper was obviously not the true systolic value. This followed from some experiments where the values obtained with the photoelectric method was compared not only with the usual mercury manometer readings but also with blood pressure recordings obtained with a membrane



manometer connected with the radial artery. It was then observed that the values from the photoelectric method corresponded to the mean blood pressure values as judged from the mercury manometer recording and not to the systolic spikes obtained with the membrane manometer.

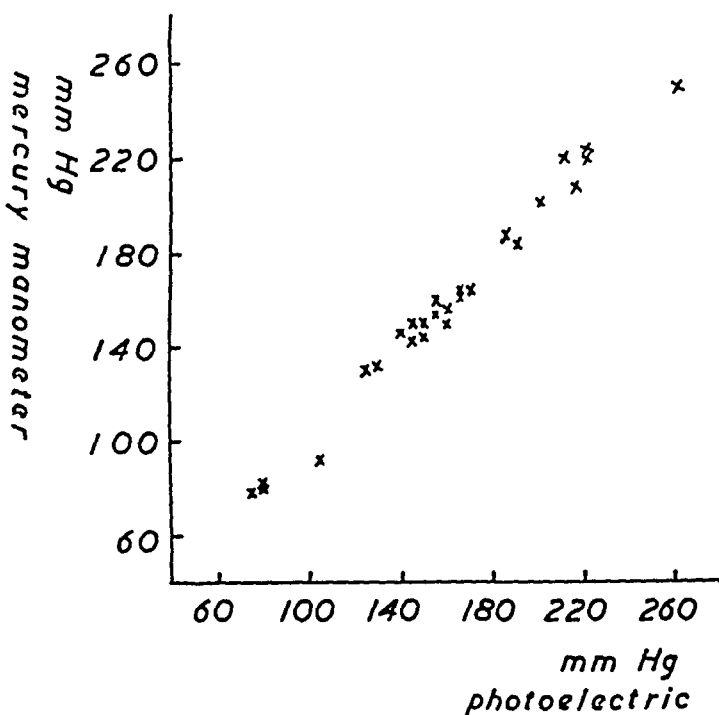


Fig. 6.

When treating the values from the two different methods statistically it was found that the mean difference between these values was  $0.46 \pm 1.07$  mm Hg (standard deviation ( $\delta_d$ ) =  $\pm 5.65$ ), a difference which is not significant. This denotes that there was no systematic difference between the two methods. Thus they can be used alternatively.

### Summary.

A new method for the estimation of the blood pressure in intact dogs is described. A cuff is applied round one of the forearms and a photoelectric cell is used to indicate the beginning of the passage of the blood stream through the distal part of the leg (where a local vasodilatation, necessary to give distinct pulsations, is caused by pricking in histamine into the skin). By means of an

amplifier and a mirror galvanometer the pulsations are projected upon a screen attached to the scale of an Hg-manometer.

When comparing the values obtained with this indirect method and the values obtained with the usual direct mercury manometer method a fairly close agreement was observed.

The method allows frequent estimations of the blood pressure for long periods in intact, unanaesthetized dogs.

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## Pharmacological Properties of Some New N-substituted Barbituric Acid Derivatives.

By

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In earlier publications it was observed that there is an obvious relationship between the pharmacological action and the chemical structure of certain barbituric acid derivatives. SWANSON and his associates (1945) conclude that in the substitution of primary or secondary alkyl groups in one of the 5—5 positions, both the median anaesthetic dose and median lethal dose become relatively smaller with an increasing number of C-atoms in the alkyl chain. When, however, the alkyl radical is longer than 5 C-atoms, the amount required for anaesthesia or death in rats again increases. The therapeutic index gradually increases, as the alkyl chain becomes longer. The duration of action becomes shorter as the alkyl chain lengthens to 6 C-atoms in the primary and to 7 C-atoms in the secondary alkyl substituted derivatives. When the alkyl radical is longer than 6 C-atoms (primary alkyls) or 7 C-atoms (secondary alkyls), the duration of action in rats again increases. The substitution of a methyl, ethyl or allyl radical on one of the nitrogens, or the substitution of a 2-methyl-allyl or a 3-methyl-allyl radical in one of the 5—5 positions, or a sulphur atom in the place of the oxygen on the 2 C-atom also obviously reduces the duration of action.

SHONLE (1931) submitted the following general rules: The sum of the carbon atoms in the two substituent groups in the 5—5 positions is seven in the most effective compounds; the greatest

dissimilarity in the two groups gives the greatest activity and if both groups are larger than ethyl, less effective compounds result.

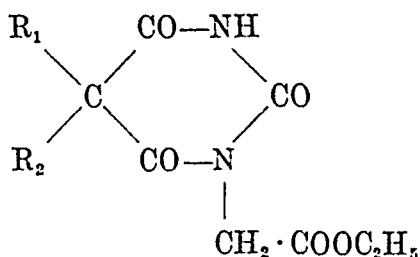
Nevertheless objections to these generalizations were raised by BUTLER and BUSH (1942) and by HUNT et al. (1946), who concluded that there are notable exceptions to any generalization regarding the relationship of chemical structure to activity.

The aim of the present paper is:

To study the relation between chemical configuration and pharmacological action of a new series of N-substituted barbiturates.

### Experimental.

The present investigation is concerned with the study of a series of 5,5-disubstituted barbituric acid derivatives synthesized by ROSÉN and SANDBERG (1949) with the general formula:



Wherein a)  $\text{R}_1 = \text{allyl}$  and  $\text{R}_2 = \text{alkyl radicals with 1 to 5 C-atoms.}$

b)  $\text{R}_1 = \text{R}_2 = \text{allyl}$

c)  $\text{R}_1 = \text{R}_2 = \text{ethyl}$

The definition of anaesthetic and hypnotic efficiency varies considerably in the literature, but should not, as will be shown below, be expressed in a single figure.

In this paper the following definitions will be used:

1. *Hypnosis* or hypnotic efficiency is defined as that condition in which it is not possible to elicit »body righting reflexes» (FULTON 1938) regardless of duration. The median hypnotic dose,  $\text{HD}_{50}$ , gives hypnosis in half the population of rats.

2. The term *narcosis* or narcotic efficiency defines the state in which an animal (rat), placed on its side, remains in that posture. Thus, hypnosis is a pre-narcotic condition.

3. *Induction time* is the time from the moment of injection until the stage of narcosis is reached.

Summary of determi  
The general formula of the new deriva

## A. Hypnotic efficiency

t

No.	Derivatives	Num- ber of rats	Slope of regression line (= b) b $\pm$ S. E.	HD <sub>50</sub> $\pm$ S. E.		Num- ber of rats	Slope of regression line (= b) b $\pm$ S. E.
				Mg/Kg	Millimols/Kg		
1	Hexobarbitone B. P. . .	72	10.2 $\pm$ 4.1	33.3 $\pm$ 1.24	0.141 $\pm$ 0.0052	186	10.6 $\pm$ 2
2	Aprobarbital N. N. R. . .	45	11.4 $\pm$ 3.9	25.2 $\pm$ 1.04	0.120 $\pm$ 0.0050	61	8.0 $\pm$ 2.9
3	methyl-allyl-derivative..	55	8.9 $\pm$ 2.9	170.5 $\pm$ 8.24	0.635 $\pm$ 0.0307	24	10.4
4	ethyl-allyl- " ..	49	13.5 $\pm$ 5.8	168.5 $\pm$ 7.75	0.597 $\pm$ 0.0274	36	24.6 $\pm$ 7.0
5	n-propyl-allyl " ..	79	11.6 $\pm$ 3.6	197.7 $\pm$ 6.05	0.667 $\pm$ 0.0204	34	8.3 $\pm$ 6.3
6	iso-propyl-allyl " ..	60	7.4 $\pm$ 4.4	179.5 $\pm$ 9.21	0.606 $\pm$ 0.0311	38	12.0 $\pm$ 4.3
7	n-butyl-allyl- " ..	80	11.6 $\pm$ 4.1	197.2 $\pm$ 5.89	0.636 $\pm$ 0.0190	40	12.5 $\pm$ 4.4
8	sec. butyl-allyl- " ..	63	9.0 $\pm$ 2.4	108.3 $\pm$ 2.76	0.349 $\pm$ 0.0089	60	13.2 $\pm$ 5.0
9	iso-butyl-allyl- " ..	65	8.2 $\pm$ 3.9	214.9 $\pm$ 10.8	0.692 $\pm$ 0.0348	43	8.7 $\pm$ 3.5
10	iso-amyl-allyl- " ..	55	6.7 $\pm$ 2.5	123.5 $\pm$ 7.47	0.381 $\pm$ 0.0230	35	14.7 $\pm$ 5.4
11	di-allyl- " ..	55	6.1 $\pm$ 1.5	96.9 $\pm$ 7.04	0.329 $\pm$ 0.0239	71	17.4 $\pm$ 4.9
12	di-ethyl- " ..	55	7.0 $\pm$ 3.6	164.4 $\pm$ 10.2	0.608 $\pm$ 0.0378		—

b = 9.303 on an average

$$\sigma = \frac{1}{b} = 0.108$$

4. *Incidence* of narcosis is the number of rats reacting with narcosis out of the total number of rats injected.

5. *Duration of action* is the time from the inception of narcosis until the rat assumes a sitting posture.

6. *Therapeutic index* is the ratio between the median lethal dose (LD<sub>50</sub>) and the median hypnotic dose (HD<sub>50</sub>).

## I. Methods.

a) Determination of HD<sub>50</sub>:

Healthy male albino rats of the same strain weighing 80—120 g were used. The rats were deprived of food for 18—21 hours prior to injection. For one hour before and during the first two hours of the experiments, the rats were kept at the same temperature (26° C) in the same quiet room.

Each barbiturate was dissolved in molecular amounts of N/1 sodium hydroxide and diluted with distilled water, the final solution containing 0.161 millimols per ml of the acid in the form of its sodium salt. All barbiturate solutions were freshly prepared and were never older than two hours. All injections were made intraperitoneally by the same operator. The frequency of "body righting reflexes" with three different doses was noted with at least 15 rats in each dose level. From these data the HD<sub>50</sub> was computed from the linear regression equation which re-

le I.

nation on white rats.

tives Nos. 3—12 is given on page 002.

Toxicity

C. Duration of action of  $2 \times HD_{50}$ 

LD <sub>50</sub> ± S. E.		Thera- peutic index	Num- ber of rats	Induc- tion time min.	Incidence % ± S. E.	Duration of action	
Mg/Kg	Millimols/Kg					log min. ± S. E.	Minutes ± S. E.
276.3 ± 6.1	1.169 ± 0.0258	8.3	517	1.84	98.2 ± 0.6	1.3773 ± 0.0091	23.8 ± 0.50
85.4 ± 4.5	0.406 ± 0.0214	3.4	65	5.78	100.0 ± 1.5	2.4342 ± 0.0160	272 ± 10.0
2.130	7.938	12.5	133	2.46	98.5 ± 1.1	1.2652 ± 0.0258	18.4 ± 1.09
1.000 ± 22.0	3.542 ± 0.0779	5.9	125	2.15	100.0 ± 0.8	1.3723 ± 0.0239	23.6 ± 1.30
1.101 ± 68.5	3.716 ± 0.231	5.6	117	2.22	98.3 ± 1.2	1.5030 ± 0.0276	31.8 ± 2.02
897 ± 38.8	3.027 ± 0.1309	5.0	130	2.04	98.5 ± 1.1	1.4346 ± 0.0260	27.2 ± 1.63
838 ± 36.0	2.701 ± 0.1160	4.3	140	2.36	95.7 ± 1.7	1.4634 ± 0.0270	29.1 ± 1.81
890 ± 36.0	2.868 ± 0.1160	8.2	128	2.47	87.5 ± 2.9	1.1598 ± 0.0287	14.5 ± 0.96
919 ± 48.8	2.962 ± 0.1573	4.3	140	2.27	91.4 ± 2.4	1.3618 ± 0.0322	23.0 ± 1.70
807 ± 31.2	2.488 ± 0.0962	6.5	130	3.28	89.2 ± 2.7	1.1992 ± 0.0292	15.8 ± 1.06
840 ± 18.7	2.854 ± 0.0635	8.7	134	2.66	96.3 ± 1.6	1.1971 ± 0.0274	15.7 ± 0.99
—	—	—	117	2.98	97.4 ± 1.5	1.2428 ± 0.0329	17.5 ± 1.32

b = 12.76 on an average

$$\sigma = \frac{1}{b} = 0.0784$$

lates the probit of hypnosis to the logarithmic concentration according to Finney's formula (1947). The results are summarized in Table I A.

Hexobarbitone (Evipan) was used as a reference standard. In order to control that there was no heterogeneity all animals were injected with the reference standard as well as with the test preparations. A period of 5—7 days was allowed to elapse between different injections. Under such conditions no significant difference in the susceptibility of the rats was observed and a single animal could be used for as much as 3 or 4 separate tests.

#### b) Determination of LD<sub>50</sub>:

This determination was carried out under the same conditions as for HD<sub>50</sub> with two exceptions: the concentration of the barbiturate solutions was 0.322 millimols per ml, and the time of observation during which the temperature (26° C) was thermostatically controlled was 24 hours. LD<sub>50</sub> was calculated in the same way as HD<sub>50</sub>. A survey of the toxicity of the derivatives is given in Table I B.

#### c) Determination of duration of action, incidence and induction time:

Equiactive hypnotic doses, of twice the assayed HD<sub>50</sub>, of the barbiturates were injected intraperitoneally as described above. The concentration of the solutions was 0.161 millimols per ml as in the determination of HD<sub>50</sub>. The induction time, incidence and duration of action were noted. The use of continuous observation of the duration

of action was preferred as the most adequate and the end-point could be determined with an accuracy of 5 seconds. All the rats included in these experiments were divided into 8 groups and the susceptibility of the animals was controlled by injecting Hexobarbitone into each group. Each barbiturate was injected at intervals of 5—7 days into two of these eight groups of rats. The results are shown in Table I C.

### *Statistical treatment:*

#### **I. Duration of action:**

Two facts became evident from a special analysis: The absolute values for duration of action show a continuous skew distribution with a high number of low values where narcosis was successful. The non-reactors, *i. e.* the rats, in which the injection of barbiturates failed to produce narcosis, form a group, significantly separated from the other values.

The non-reactors were excluded and the absolute values for duration of action converted into logarithms. The logarithmic values were grouped for analysis, since the number of individuals in each series was rather large (52—79) with class intervals equally large in terms of the logarithm of time. In each case the class interval includes its upper but not its lower limit. The grouped frequencies were then accumulated and transformed into percentages and probits. Finally each probit was plotted against its corresponding upper class limit. These cumulative curves became approximately rectilinear. See Figure 1.

According to BLISS (1937) the function of time against which the probit can be plotted as a straight line is distributed normally. Thus, in this case the logarithm of the time for duration of action is distributed normally and in the following all calculations were performed on logarithmic values with the aid of ordinary statistical methods. The non-reactors were excluded and treated separately (*cf.* below: 2. Incidence). The average of logarithmic values corresponds to a geometric mean. The value for duration of action given in Table I C is the average of two means, determined separately in the two groups of rats used for each compound. The standard deviation and the standard error of the mean in each determination were calculated according to the formula of COCHRAN (1938). The coefficient of variation in these determinations was 37.0 per cent on an average with the range 25.8—48.1 per cent. The standard error given in Table I C was computed as follows:  $S. E. = \sqrt{e_1^2 + e_2^2}$  where  $e_1$  and  $e_2$  are the standard errors of each determination.

#### **2. Incidence:**

A graphic analysis of the average durations of action and incidences for the different derivatives showed that there was no correlation between duration of action and incidence. Nor was there a correlation between incidence on one side and  $HD_{50}$  and the slope of its regression line on the other. Thus, duration of action and incidence are independent qualities. This justifies the calculation of each of these different properties. The statistical significance of the incidence, if expressed as an

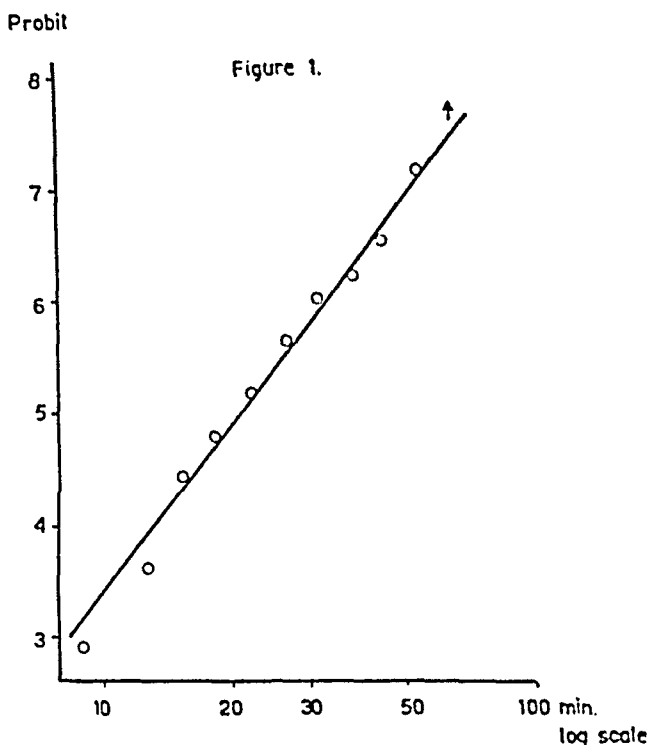


Fig. 1. Probit transformation of the logarithmic times for duration of action from a group of 77 rats treated with 66.6 mg of Hexobarbitone per kg of bodyweight ( $2 \times \text{HD}_{50}$ ). In this group there were 2 non-reactors. The slope of the line is  $5.22 \pm 1.25$  and Chi-square for 9 degrees of freedom was found to be 0.53 giving  $P > 0.9$ , i. e. no evidence of heterogeneity of departure from the fitted probit line was found.

incidence  $I$  in per cent, is computed from the standard error of the incidence, S. E.

$$\text{S. E.} = \pm \sqrt{\frac{I(100-I)}{x}}$$

where  $I$  = incidence in per cent

$x$  = total number of animals

The significance of a single value for incidence may be tested by  $t$ -analysis. The value of Incidence in Table I C is calculated from both groups of rats taken as a whole.

### 3. Induction time:

The mean is calculated by the ordinary statistical methods from the logarithmic values of time of both groups of animals taken as a whole. A graphic probit analysis showed that these transformations gave approximately straight lines. Owing to the relatively slight importance of this property, the standard error of the mean was not computed throughout. In two cases it was calculated and the values were  $\pm 2.2$  and  $\pm 2.3$  per cent.



## II. Results and Discussion.

A special statistical analysis clearly showed that there was no correlation in these experiments between the properties: potency, toxicity, incidence, duration of action and induction time. This is also evident from even a hasty glance at Table I. Therefore, in the following these properties must be treated separately since they are independent qualities.

GOLDBERG (1947) and BJÖRN (1947) have shown the same independence between similar qualities when testing local anaesthetics. It would therefore appear that, in order to obtain a clear evaluation of a local anaesthetic or a hypnotic, regard must be paid to more qualities simultaneously than has usually seemed necessary.

A homologous series of derivatives, as in this case, affords the opportunity of comparing the relative influence of a single radical on pharmacological properties. Thus we are able to compare the relative potency of the alkyl radicals on one of the 5—5 positions. The significance of a difference between two median hypnotic doses may be tested by t-analysis.

For a study of the *potency* (Table I A) of the new derivatives the median hypnotic doses, expressed in millimols per kilogram may be compared. None of the new compounds has the potency of Hexobarbitone. The diallyl derivative (No. 11), corresponding to 43 per cent of the potency of Hexobarbitone has the greatest potency; the iso-butyl-allyl derivative (No. 9), corresponding to 20 per cent of the potency of Hexobarbitone, has the lowest.

Roughly speaking, the new N-substituted barbiturates can be divided into two main groups with reference to their potencies. The di-allyl-, sec.-butyl-allyl- and iso-amyl-allyl-derivatives have approximately twice the potency of the other members of this series.

In the derivatives Nos. 3, 4, 5, 7 the number of C-atoms in the alkyl chain increases from one to four (the next two homologues: n-amyl-allyl- and n-hexyl-allyl-derivatives did not crystallize and were accordingly not examined), but there is no regular increase in the potency as has been claimed for other types of barbiturates. Only between the n-propyl and ethyl radicals is there a probable difference, the ethyl radical, however, having the greatest potency. There is probably no difference between the propyl isomers.

In the butyl-substituted isomers, the sec.-butyl derivative has the greatest potency and the iso-butyl derivative has the lowest. This result is in agreement with an observation of SWANSON and FRY (1940). However, the difference between the isobutyl and n-butyl derivatives is not probable ( $P = 0.3-0.1$ ). On the other hand, the difference between the sec.-butyl and n-butyl derivatives is highly significant ( $P < 0.001$ ).

There is no probable difference between the iso-amyl-allyl-, sec.-butyl-allyl- and diallyl-derivatives, forming a group of the greatest potency.

The derivatives Nos. 12, 4 and 11 will now be compared:

di-ethyl-der.	ethyl-allyl-der.	di-allyl-der.
HD <sub>50</sub> : 0.608	0.597	0.329

In the di-ethyl- and ethyl-allyl-derivatives the ethyl and allyl radicals have approximately the same relative potency, but if the ethyl-allyl- and di-allyl-derivatives are compared the allyl radical has about twice the relative potency of the ethyl radical.

Thus, in one case two radicals (ethyl and allyl) have the same relative potency, in another case one of them has twice the potency of the other. This example clearly shows that the rules of the influence of a single radical upon potency are not generally applicable.

In terms of millimols per kilogram Hexabarbitone has more than twice the *toxicity* (Table I B) of the iso-amyl-allyl derivative (No. 10), which is the most toxic of the new substances (Nos. 3-12). The methyl-allyl derivative has the lowest toxicity. In order to control the unexpectedly high value of its LD<sub>50</sub> (2130 mg/kg, determined from two dose levels) the following doses were injected: 750 mg/kg into 6 rats, 900 mg/kg into 12 rats, 1,000 mg/kg into 12 rats, 1,100 mg/kg into 7 rats and finally 1,500 mg/kg into 10 rats. All animals survived.

The amount of the diethyl derivative (No. 12) required for the determination of LD<sub>50</sub> was not available. Only a single dose of 900 mg/kg was injected into 12 rats, 5 of which died.

There is a probable difference ( $P = 0.02-0.01$ ) between the propyl isomers, the isopropyl derivative being the most toxic, but no probable difference in toxicity between the butyl isomers — contrary to the case with their potencies.

As mentioned above, the isoamyl-allyl derivative is the most toxic; if compared with the diallyl derivative there is a signif-

icant difference ( $P = 0.003-0.001$ ) and if compared with the ethyl derivative a highly significant difference ( $P < 0.001$ ).

In regard to *therapeutic index* (Table I B) a single derivative — methyl-allyl- (No. 3) with a therapeutic index of 12.5 — differs obviously from the others, mainly owing to its low toxicity. The sec.-butyl-allyl- (No. 8) and di-allyl- (No. 11) derivatives have about the same value of therapeutic index as Hexobarbitone *i. c.* 8.3. All the others have a therapeutic index between 4.3 and 6.5. SWANSON and coworkers (1945) claim that the therapeutic index increases as the alkyl chain lengthens. This is not, however, the case in this series.

With reference to *duration of action* (Table I C) the new derivatives may be divided into three main groups:

1. Those with a shorter duration of action than Hexobarbitone: sec.-butyl-allyl-: 14.5 min., diallyl-: 15.7 min., iso-amyl-allyl-: 15.8 min., diethyl-: 17.5 min., methyl-allyl-: 18.4 min.

2. Those with the same duration of action as Hexobarbitone: isobutyl-allyl-: 23.0 min. and ethyl-allyl-: 23.6 min.

3. Those with a longer duration of action than Hexobarbitone: iso-propyl-allyl-: 27.2 min., n-butyl-allyl-: 29.1 min., n-propyl-allyl-: 31.8 min.

The figures give the average duration of action of  $2 \times HD_{50}$ .

#### First group:

There is no probable difference ( $P > 0.05$ ) between the sec.-butyl-allyl-, diallyl- and isoamyl-allyl-derivatives, and these compounds are, as far as the writer is aware, the most ultra short-acting barbiturates hitherto examined, with a duration of action equal to 61—66 per cent of that of Hexobarbitone. There are no probable differences either between the diethyl- and methyl-allyl-derivatives, or between the diethyl- and sec.-butyl-allyl-derivatives; but the difference between the sec.-butyl-allyl- and methyl-allyl-derivatives is very probable ( $P = 0.01-0.003$ ).

#### Second group:

There is no probable difference between the two derivatives belonging to this group. If we compare the iso-butyl-allyl-derivative with the last member of the first group, the methyl-allyl-derivative, the difference is probable ( $P = 0.05-0.02$ ).

#### Third group:

Within this group there is no probable difference. Compared with a member of the second group, the ethyl-allyl-derivative,

we find no probable difference between it and the iso-propyl-allyl-derivative, but a probable one ( $P = 0.02-0.01$ ) between the former and the n-butyl-allyl-derivative. Finally, the following three compounds will be compared (cf. page 211):

	di-ethyl-der.	ethyl-allyl-der.	di-allyl-der.
Duration of action:	17.5	23.6	15.7 min.

If the di-ethyl- and ethyl-allyl-derivatives are compared the ratio of the relative duration of action between the ethyl and allyl radicals is 0.74, if on the other hand the ethyl-allyl- and di-allyl-derivatives are compared the ratio of the relative duration of action between the ethyl and allyl radicals is 1.50.

This example distinctly shows that there are no general rules for the influence of a single radical upon the duration of action of barbiturates.

Concerning the *incidence* (Table I C) we find two main groups-

1. Low incidence: sec.-butyl-allyl- 87.5 per cent, iso-amyl-allyl: 89.2 per cent and isobutyl-allyl- 91.4 per cent. Within this group there is no probable difference.

2. High incidence: n-butyl-allyl- 95.7 per cent, di-allyl- 96.3 per cent, di-ethyl- 97.4 per cent, n-propyl-allyl- 98.3 per cent, iso-propyl-allyl- 98.5 per cent, methyl-allyl- 98.5 per cent and ethyl-allyl- 100.0 per cent. Within this group we find a probable difference ( $P = 0.05-0.02$ ) between on one hand the ethyl-allyl-derivative and on the other the n-butyl-allyl- and di-allyl-derivatives. Between the ethyl-allyl-derivative and down to and including the di-ethyl-derivative there is, however, no probable difference.

A comparison between a member of the first group, the iso-amyl-allyl-derivative, shows a difference between it and the ethyl-allyl-derivative which is highly significant ( $P < 0.001$ ).

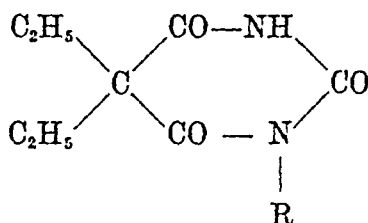
The new substances have approximately the same *induction time* (Table I C) as Hexobarbitone. With a dose of  $2 \times \text{HD}_{50}$ , the induction time is 2.49 minutes on an average with a range of 3.28 to 2.04 minutes, the value for Hexobarbitone being 1.84 minutes.

The narcosis produced in rats by all these new compounds except the di-ethyl-derivative (No. 12) was as quiet as that produced by Hexobarbitone. Except for a fine rapid tremor of the legs, which was sometimes seen in the light stages of narcosis, the rats were well relaxed.

Curiously enough and *a priori* unpredictable from the point of view of chemical structure, the di-ethyl-derivative (No. 12) is a convulsant. The convulsions are prenarctic, rather violent and clonic in type.

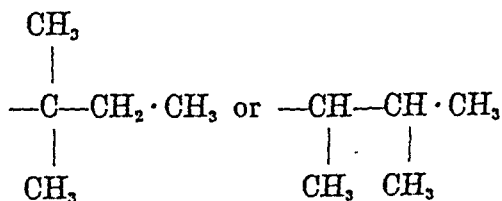
It is interesting to note that according to HJORT et al. (1939) three isomers of 1-amyl-5,5-diethylbarbituric acids show convulsant tendencies; namely 1-sec-butylcarbonyl-5,5-diethylbarbituric acid and 1-dimethylethylcarbonyl-5,5-diethylbarbituric acid reveal convulsant properties, but they also induce true narcosis just as derivative No. 12. On the other hand 1-isopropylmethylcarbonyl-5,5-diethylbarbituric acid is a pure convulsant without narcotic efficiency.

From the above observation, in conjunction with the findings of HJORT and coworkers, it is evident that the structure



gives convulsant properties, wherein R may hitherto be the radicals:

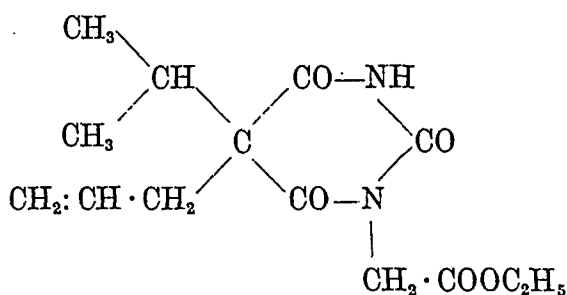
$-\text{CH}_2 \cdot \text{COO C}_2\text{H}_5$  (derivative No. 12),  $-\text{CH}_2 \cdot \text{CH} \cdot \text{CH}_2 \cdot \text{CH}_3$ ,



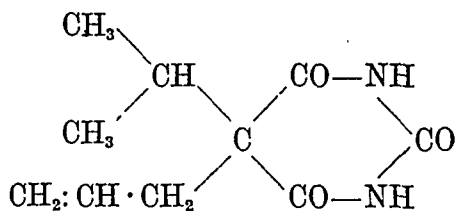
No side effects were observed with doses up to  $2 \times \text{HD}_{50}$  — except for the di-ethyl-derivative, whose convulsant properties are described above — but in toxic doses side effects were noted in two cases. In the determination of  $\text{LD}_{50}$  for the di-allyl-derivative (No. 11) 3 rats from 31 surviving showed a paralysis of the hind part of the body. Corresponding figures for the n-propyl-allyl-derivative (No. 5) were 3 paralysed rats out of 24 surviving. Of these 6 paralysed rats, 2 recovered in a day; the other 4 animals were still paralysed during the following 4—5 days, after which they were killed.

Some information on the metabolic fate of these new substances in the animal body may be obtained from a comparative study of the figures in Table I. The extremely low toxicity of the methyl-allyl-derivative (No. 3) in comparison with the other members of this series suggests the possibility that the first step in the degradation of this structure may be oxidation or demethylation of the radicals in the 5—5 position, forming a 5-mono-substituted, non-hypnotic derivative.

This hypothesis is supported by a comparison between the properties of the iso-propyl-allyl-derivative (No. 6) and Aprobarbital (No. 2) the formulae of which are:



Derivative No. 6



Aprobarbital

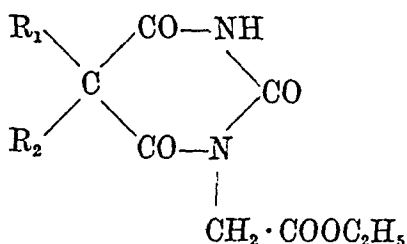
If the loss of the radical  $\text{—CH}_2\text{COOC}_2\text{H}_5$  were the principal reaction responsible for the inactivation of derivative No. 6, the residual hypnotic efficiency of Aprobarbital would be conspicuous. As Aprobarbital has five times the potency of derivative No. 6 and has ten times longer duration of action, it is very unlikely that Aprobarbital is an intermediate metabolite of derivative No. 6.

In summarizing the above results of the relation between chemical structure and pharmacological action, it can be stated that no definite relationship was found in this series. The rules found for some other types of 5,5-disubstituted barbituric acids are not generally applicable. The writer wishes to stress the opinion of BUTLER and BUSH (1942) that it is doubtless these

generalizations, that have discouraged further investigation of new barbiturates.

### Summary.

In a new series of ultra short-acting barbiturates of the general formula



no definite relationship was found between chemical structure and pharmacological efficiency in rats. There was no correlation between the properties potency, toxicity, duration of action, incidence and induction time. They are independent qualities and must be treated separately.

The logarithm of the time for duration of action showed normal distribution and all calculations were performed on logarithmic values. The non-reactors formed a group, significantly separated from those where narcosis succeeded.

Hexobarbitone (Evipan) was used as a reference standard and it was injected into all animals to control that there was no heterogeneity.

The significance of the differences in the qualities examined between two derivatives was tested by t-analysis.

Table I gives the details of the results obtained.

I wish to express my appreciation to Professor H. RYDIN for giving me the opportunity of carrying out part of this investigation in his laboratory and to his Staff for technical assistance. I am also indebted to Dr. Å. LILJESTRAND for some valuable discussions on the statistical problems in this work.

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## Protein Metabolism of Tissue Cells in Vitro. 7.

### The Chemical Nature of Some Obligate Factors of Tissue Cell Nutrition.

By

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From the works of the last ten years on cultivation of tissue cells *in vitro* on more or less artificial media (partly synthetic), some common features could be recognized as to special importance of certain low-molecular substances. The works of FISCHER et al. since 1941 and later WHITE (1946) clearly indicate that the low-molecular substances that form the basal requirements for the cells are to be found under the following headings:

organic phosphates,  
sugars,  
amino acids,  
peptides.

Of the substances belonging to the first of these groups several organic phosphates have been tested as to their effect on growth of fibroblasts *in vitro*, for example fructose-1,6-diphosphate, adenosinetriphosphate,  $\beta$ -glycerophosphate and amino-ethanol phosphoric ester. It seems likely that among these different organic phosphates the hexosediphosphate would occupy a position as the most versatile compound in biochemical respect because most other organic phosphates can be formed from this compound in the course of normal carbohydrate breakdown in the tissue

cells. Thus for instance the formation of ATP would be the result of hexose metabolism in the presence of a phosphate source and so far no special effect of ATP added to growing cultures of tissue cells has been observed (see below). Assuming that the main pattern of carbohydrate metabolism in tissue cells can provide a steady output of organic phosphates with energy-rich phosphate bonds like ATP — the transfer of phosphate to other organic compounds would be a consequence.

Thus hexosediphosphate fulfils the double function of a basic nutritional component providing the cells with organic bound phosphate and a suitable carbohydrate, both of which form the basis for furnishing the necessary energy for the metabolism of the cells. As to be shown later in this paper some sugars together with a suitable phosphate source could under certain circumstances replace hexosediphosphate as nutritional component, but there is *a priori* no reason to assume that the metabolism of these components would run along other lines than over the hexosediphosphate breakdown pattern.

As to the amino acid group the experiments of FISCHER (1941) indicated that, first, amino acid mixtures, added to dialyzed medium, could maintain for a shorter time a certain amount of growth, provided that the amino acids were present in the same proportion as in fibrinogen. Second, that cystine keeps a key position among the amino acids tested. In this connection it is to be recalled that FISCHER (1941) found that methionine could not replace cystine in fibroblast cultures, probably due to defect of the demethylation mechanism. Two other members of the amino acid group have later been shown to be obligate for the cultivation of chicken fibroblasts, namely glycine and glutamine. The first of these components, glycine, is shown by several workers, *e. g.* ALMQUIST and coworkers (1940), HEGSTED et al. (1941) to be a necessary factor for nutrition of chickens, and its importance for chicken fibroblast culture is lately confirmed by FISCHER (1948 a). Furthermore recent experiments of SONNE et al. (1946) and SHEMIN and RITTENBERG (1947) have shown that glycine most probably is one of the components involved in purine synthesis and it is therefore necessary to be aware of the possibility that glycine and purines may be interchangeable to a certain degree concerning cell nutrition. Glutamine on the other hand is recently shown by FISCHER (1948 b) to have a curative effect on atrophic cells growing in deficient media. The effect of glutamine

on atrophic glass-splinter-like cells, which often could be found in cultures on synthetic insufficient media, is striking in its rapid change of the morphological picture, providing apparently quite normal cells.

There remains the diffuse and never explained effect of "peptides" under which heading we find different preparations, such as enzymatic digests of proteins, from different sources, and some commercial preparations like "Witte peptone". The common feature of all these products of partial protein break-down is that they contain some fractions, which have a marked influence on cell proliferation, for the first time shown by BAKER and CARREL (1928). Previous experiments have shown that of the entire spectra of peptides — from simple dipeptides up to "proteoses" — the components belonging to the trichloroacetic acid soluble fractions and at the same time belonging to the non-dialysable fractions are the most active in this respect. Thus it may be concluded that we have to seek for the components possessing maximal activity in respect of the induction of cell proliferation between some special definite limits of molecular weight, namely the upper limit of trichloroacetic acid precipitability and the lower limit of non-dialyzability.

In the light of all these experiments, it seems to us necessary to make a detailed investigation about the effect of a simplified medium for maintenance and growth of tissue cells based on the presence of some of the compounds discussed above, that is: glucose and a suitable phosphate source, glycine, cystine, and glutamine, leaving the peptides outside the question for the time being. Of these four components we have taken each one, making replacement experiments using the technique, previously described by FISCHER (1941). As will be shown, the presence of these four components is an absolute necessity for maintenance and growth of tissue cells, and it will be also shown that they occur in "natural" nutritional media, based on dialyzates from plasma and embryo extract. It may be pointed out that all the factors discussed belong to the dialysable low-molecular substances occurring in nature as the nutritional components of cells from plants and animal tissues. At this stage we might stress that we are fully aware of the danger of oversimplification, but that the investigation of the few basal components of cell nutrition will provide the basis for further improvements of similar synthetic media.

# Section I.

The details of the technique employed have been described several times (FISCHER 1941), the main features involving observation of the differential growth of two halves of the same fibroblast culture from embryonic chicken heart. The preceding development of these cultures involves from 5 to 20 transfers in non-dialyzed media using the hanging drop method. The medium for the following cultivation on dialyzed media was of the type:

<i>Solid phase</i>	<i>Fluid phase</i>
0.5 cc dial. plasma	0.5 cc dial. serum
1.0 cc Tyrode solution	0.1 cc X
0.1 cc X	
clotted by adding 1 drop of dialyzed embryo juice	3 drops of dial. em- bryo juice

where X represents a solution of nutritional components to be investigated. The composition of these solutions X are described in the tables appearing below. The cultivation was done in Carrel flasks, type D.3.5 at 38°. Each culture was run along with its control, the latter containing a basal diet of the four components: Fructose-1.6-diphosphate Ca-salt, glutamine, glycine and cystine, whereby each experiment implies the replacement of one of these components against a number of different substances. The pH-value was adjusted to pH 7 by introducing a gas mixture consisting of 80 per cent O<sub>2</sub>, 12 per cent N<sub>2</sub> and 8 per cent CO<sub>2</sub>. The area growth was measured by projection of the cultures by means of an Edinger projector with a magnification of 20 times. The area of each drawing was measured by means of a planimeter and the results are expressed as ratios according to the formula E/C where E is the area of a culture after a certain period of time, and C the area of the corresponding control.

The following tables show the results of the placements of each one of the four components mentioned above.

As a commentary to the above results it seems that any phosphate source together with glucose could replace fructose-diphosphate. The improved growth of cultures after addition of inorganic phosphate as compared with controls without, means that the phosphate content of the Tyrode solution is sufficient (ca. 50 mg. pr. liter). That inorganic phosphate + glucose thus seems to work like the hexosediphosphate is only an indication of an intact carbohydrate metabolism of the tissue cells. The reason why aminoethanol phosphoric ester was tested was that it recently had been found in embryonic extracts (GORDON, 1949 in press). In fact, as phosphate source this substance seems to work somewhat better than inorganic phosphate, and may in the embryonic extract function as a suitable phosphate source.

Table 1 a.

*Exchange of Phosphates.*

Standard components in 10 cc		Ratio of growth $\frac{E}{C}$			
		Days			
		1	2	3	4
Fructose-diphosphate Ca-salt .... 30 mg					
Glutamine ..... 25 "					
Glycine ..... 25 "					
Cystine ..... 1.5 "					
Fructose-1.6-diphosphate replaced against					
E 1	Glucose ..... 12 mg	0.9	0.8	0.7	0.5
No phosphate addition					
E 2	Glucose ..... 12 mg	1.0	1.0	1.0	1.0
	$\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$ ..... 40 "				
E 3	Aminoethanol phosphoric ester ..... 12 mg	1.1	1.1	1.1	1.1
	Glucose ..... 12 "				
E 4	ATP ..... 10 mg	0.9	1.0	1.0	1.0
	Glucose ..... 12 "				
E 5	Glycerin-phosphoric ester ..... 20 mg	1.0	1.0	1.0	1.0

Table 1 b.

*Exchange of Sugar.*

Standard components in 10 cc		Ratio of growth $\frac{E}{C}$			
		Days			
		1	2	3	4
Glucose replaced against					
E 1	$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ ..... 40 mg	0.9	0.8	0.7	0.5
No sugar addition					
E 2	Aminoethanol phosphoric ester ..... 12 mg	1.1	1.2	1.4	1.5
	Sorbose ..... 20 "				
E 3	Aminoethanol phosphoric ester ..... 12 mg	1.0	1.0	1.0	1.0
	Sorbitol ..... 20 "				
E 4	Aminoethanol phosphoric ester ..... 12 mg	0.8	0.7	0.7	0.6
	Ribose ..... 20 "				
E 5	Aminoethanol phosphoric ester ..... 12 mg	0.9	1.0	1.0	1.0
	Rhamnose ..... 20 "				

In the experiments with different sugars the Tyrode-solution used was glucose-free. As to the exchange of the fructose rest in hexose-diphosphate against various sugars, sorbose and the

Table 2.

*Exchange of Glycine.*

Standard components in 10 cc		Ratio of growth $\frac{E}{C}$			
		Days			
		1	2	3	4
Fructose-disphosphate Ca-salt ...	30 mg				
Glutamine .....	25 "				
Glycine .....	25 "				
Cystine .....	1.5 "				
Glycine replaced against					
E 1	No glycine .....	0.7	0.6	0.5	0.5
E 2	1 + alanin ..... 30 mg	1.0	1.0	1.1	1.1
E 3	dl serin ..... 70 mg	1.0	1.0	0.9	0.8
E 4	Glycyl-glycin ..... 25 mg	1.0	1.0	1.0	1.0
E 5	Sarcosine ..... 20 mg	0.9	0.8	0.7	0.6
E 6	$\beta$ -alanine ..... 30 mg	0.9	0.9	0.8	0.8
E 7	Glycine ..... 25 mg	1.1	1.1	1.2	1.2
	Hypoxanthine ..... 1.5 "				
E 8	Adenine ..... 1.5 mg				
	Guanine ..... 1.5 "	1.0	1.0	1.0	1.0
	Hypoxanthine ..... 1.5 "				

The same value was found with tri-, tetra-, penta- and hexaglycine.

corresponding sorbitol seems to function in the presence of an organic phosphate source, which could be explained on the basis that the cells are able to convert these sugars to components of normal glucose breakdown. Earlier works with various sugars (ASTRUP, FISCHER and ÖHLENSCHLÄGER (1947)) indicate that glucose could be replaced by mannose and to some extent by galactose. The negative action of ribose in the presence of organic phosphates is not surprising considering the different pathways of metabolic breakdown of hexoses and pentoses. Other experiments by ASTRUP et al. (1947) show that no pentose is metabolized by tissue cells even in complex media.

Considering the above results it seems somewhat surprising that alanine could replace glycine, because no experiment with labelled alanine have shown any direct connection with glycine (EHRENSVÄRD et al. 1948). The results with the glycine peptides speak for themselves, but the experiments with the purine mixture need a special comment. It seems that of the purines tested hypo-

Table 3.

*Exchange of Glutamine.*

Standard components in 10 cc		Ratio of growth $\frac{E}{C}$				
		Days				
		1	2	3	4	
Fructose-disphosphate Ca-salt	30 mg					
Glutamine	25 "					
Glycine	25 "					
Cystine	1.5 "					
Glutamine replaced against						
E 1 No glutamine		0.9	0.7	0.6	0.5	
E 2 Asparagine	22 mg	1.0	1.0	1.0	1.0	
E 3 $H_2NNO_3$	14 mg	0.9	0.7	0.6	0.5	

Table 4.

*Exchange of Cystine.*

Standard components in 10 cc		Ratio of growth $\frac{E}{C}$				
		Days				
		1	2	3	4	
L-cystine replaced against						
E 1 No cystine		No growth				
E 2 1 + cysteine	1.5 mg	0.7	0.6	0.5	0.4	
E 3 Taurine	1.5 mg	No growth				
E 4 Cysteic acid	1.5 mg	No growth				
E 5 Cysteinyl-glycine	5 mg	No growth				

xanthine alone + the standard components increased the area of growth and had a marked influence on the rate of mitoses. This action of hypoxanthine recalls the experiments by HOPKINS (1944—1945) with myoblasts on non-dialyzed media. Without glycine hypoxanthine did only show this effect in the presence of adenine and guanine. The reason for this, and for the inactivity of all other purines tested, is at present unknown and may be revealed by further experiments connected with the biosynthesis of purines.

The rather strict specificity of the action of glutamine and cystine is emphasized by the fact that only asparagine could replace the former and (partly) glutathione the latter (ASTRUP and

FISCHER 1946). It seems that the requirement of the amide group in glutamine (or asparagine) is connected with an inability to form this grouping from glutamic acid, ammonia and ATP as outlined by SPEK (1947). It is interesting to recall that the cystine-cysteine system activates the formation of glutamine, apparently one of the many functions of this redox system.

Reviewing the above experiments we find that of all the components tested no one has an improving action on the mixture of the four components: hexose-diphosphate, glutamine, glycine and cystine under the experimental condition employed, except hypoxanthine, which may therefore be added to the list. The action of these five components is however only tested for a shorter period of time, and further long range cultivation would reveal nutritional defects of this medium. In order to put the 5-components medium to a harder test cultures of embryonic chicken fibroblasts were cultured in this medium using the hanging drop method, whereby the tissue was transferred five times without visible signs of deficiency growing at the same rate as the corresponding cultures in Carrel flasks. The total composition was the following:

For Carrel-flasks	Fructose-1.6-diphosphate Ca-salt . . . . .	30 mg
	Glutamine . . . . .	25 »
	Glycine . . . . .	25 »
	Cystine . . . . .	1.5 »
	Hypoxanthine . . . . .	0.7 »

dissolved in 10 cc Ringer solution. Sterile filtered before use.

Total medium with plasma and extract:

For hanging-drop	3 ml dial. plasma
culture.	0.3 ml 5-components mixture
	1 dr. dial. embryo juice

The above list of the 5-components would be of only academic interest if not these substances could be found in natural nutritional media, such as embryo extract, serum, malt extract and others. The next section of this paper contains an analysis of media, which clearly indicates the presence of the hexose-phosphate system, the amino acids and hypoxanthine discussed above.

## Section II.

The use of partition chromatography on paper (CONSDEN, GORDON and MARTIN 1944) has been of great value as an analytical tool for investigating the different components of tissue cell



nutrition. From the extensive work by GORDON (1949) on the content of the intercellular fluid of calf embryo muscle, it can be found that glycine, glutamine and aminoethanol phosphoric ester are present in large quantities, all the corresponding spots registered as "strong". Cystine, on the other hand, does not appear in Gordon's spot test experiments, but has been found as cysteic acid by oxidation of the test solution with bromine water. A paper chromatogram of the oxydized mixture from calf embryo muscle extract reveals a spot of cysteic acid.

As to the sugars, the method of PARTRIDGE (1948) indicates the presence of reducing hexoses (glucose). Free hypoxanthine is found by a modification of Chargaff's method of purine chromatography (VISCHER and CHARGAFF, 1947).

The method employed involved one-dimensional paper chromatography in *n*-butanol + 20 per cent glacial acetic acid saturated with water. After drying the spots were developed by dipping the strip into a solution of 0.3-N  $\text{AgNO}_3$  in 10 per cent ammonia solution and subsequent washing with, at first, water, then 0.01 per cent ammonia and at last water again. After this treatment the strip was dipped into 10 per cent formaldehyde solution and then dried at 110° C. The presence of hypoxanthine is shown by a yellow spot moving faster than other purines, except adenine. The following table (Table 5) shows some  $R_F$ -values of the purines by this method.

Table 5.

Adenine .....	0.63
Guanine .....	0.44
Hypoxanthine .....	0.52
Xanthine .....	0.50
Uric acid .....	0.38

In this connection it may be mentioned that GORDON, *loc. cit.* (1949) has confirmed from the same material the above findings by identifying the absorption spectrum of hypoxanthine with aid of the Beckman photometer.

Thus the nutritional components: Reducing hexoses + phosphates, glycine, cystine, glutamine and hypoxanthine have all been established to be definite members of the contents of the dialysate from calf embryo extract, which in turn has earlier been shown by ASTRUP, EHRENSVÄRD, FISCHER and ÖHLEN-SCHLÄGER (1947) to constitute a fully complete set of accessory growth substances. Considering the results of the first section of this paper concerning growth experiments on a simple medium

containing the substances mentioned, there seems to be conclusive proof that these compounds constitute the basal diet of chicken fibroblasts, cultivated *in vitro*. But to push the question further a direct comparison of two-dimensional paper chromatograms from dialysates of calf embryo muscle extract and an adult hen show a nearly identical picture with the only exception of the occurrence of hydroxylysin phosphate (GORDON, 1948) in the former. Thus the five components system discussed may not only be the basal nutrition for chicken fibroblasts *in vitro* but also constitute an important part of the nutritional environment of the cells of embryonic chicken tissue.

At this stage it is of interest to correlate the above findings with the results published by ASTRUP et al. (1947) on the fractionation of calf embryo dialysate with Ba-salts, where only one of the fractions show any activity as accessory substance for chicken fibroblast cultures. Using the same technique of fractionation with the exception that ethanol was substituted against methanol we have later (unpublished results) prepared four fractions by stepwise precipitation of the Ba-salt solution with methanol. Paper chromatograms of these fractions (1. 2. 3. 4.) show that the only active one (2) contains phosphate, organic or inorganic, reducing sugars, amino acids, among which cystine was found. The inactive fraction (1) contained only phosphates. The likewise inactive fractions (3) and (4) were lacking in cystine, and cystine + phosphate respectively. Together these findings complete the picture outlined about the necessity of presence of cystine and other amino acids, sugars and phosphates. In addition the active fraction (2) contained a large proportion of its nitrogen as glutamine and purines.

### Discussion.

It may be said that the general trend in the development of synthetic media for the nutrition of tissue cells *in vitro* during the last decade has been directed towards more and more composite mixtures. The underlying thought has been that an attempt had to be made to *include*, as fully as possible, all those nutritional factors present in "natural media" — the intercellular fluids — with the hope that our present knowledge of the body constituents of animal tissue would cover the main part of those. The nutritional

schemes of CARREL and LINDBERG (1938), WHITE (1946) and FISCHER, ASTRUP, EHRENSVÄRD and ÖHLENSCHLÄGER (1948) are typical in this respect. When compared with the results obtained by GORDON (1949) from paper chromatography of dialysate from calf embryo muscle, adult beef and adult serum, it appears that some of the composite synthetic media come rather close to the composition of "natural media" in qualitative respect. Now the question arises: After it has been shown that tissue cells could be cultivated *in vitro* on a synthetic mixture of low-molecular compounds in the presence of dialyzed serum + plasma, should the future attempts in this field be pushed towards producing nutritional mixtures more and more similar to the corresponding "natural media", and what will the gain of this be? Our personal opinion is that such a development would not as such be effective in solving what is the main problem: The main features of the metabolism of individual types of tissue cells *in vitro* and *in vivo*. One has to pay attention to the fact that only part of the low-molecular components existing in the intercellular space of tissue represent substrate and necessary catalysts, a large fraction of what is found by analytical methods representing intermediary products of metabolism, slowly dialyzing out from the cells, waste products and compounds with no or insignificant influence on the biochemical system in question. Even if all of these compounds make up the intercellular environment, the practical aspects of tissue cell cultivation *in vitro* demand a sifting out of those main components, which are the chief sources of protein syntheses. The results described in this paper show that the sifting out of some hexoses + phosphates, glutamine, glycine, cystine and hypoxanthine from the complex mixtures — artificial or natural — and combining them to a simple medium is fully sufficient to maintain and keep up growth of tissue cells for a considerable period. These findings might be of considerable practical use, because of the possibilities of correlating tissue culture work with tracer experiments based on labelling one of the compounds mentioned. In addition, using the 5-component medium it might be possible to detect special differences in the nutritional pattern between different types of tissue cells, normal and pathological, adjusting the composition with eventual adding of other single components.

In this connection we intend to make a closer investigation of the mitosis-stimulating effect of higher peptides.

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Figure 1. The effect of the concentration of the *Agrobacterium* suspension on the transformation efficiency of *Agrobacterium* strains. The concentration of the *Agrobacterium* suspension was 10<sup>6</sup> cells/ml (A), 10<sup>7</sup> cells/ml (B), 10<sup>8</sup> cells/ml (C), and 10<sup>9</sup> cells/ml (D). The concentration of the *Agrobacterium* suspension was 10<sup>6</sup> cells/ml (A), 10<sup>7</sup> cells/ml (B), 10<sup>8</sup> cells/ml (C), and 10<sup>9</sup> cells/ml (D). The concentration of the *Agrobacterium* suspension was 10<sup>6</sup> cells/ml (A), 10<sup>7</sup> cells/ml (B), 10<sup>8</sup> cells/ml (C), and 10<sup>9</sup> cells/ml (D). The concentration of the *Agrobacterium* suspension was 10<sup>6</sup> cells/ml (A), 10<sup>7</sup> cells/ml (B), 10<sup>8</sup> cells/ml (C), and 10<sup>9</sup> cells/ml (D).

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## Serum Protein Concentration and Relative Albumin Percentage in Normal Individuals.

By

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Received 1 April 1949.

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The determination of the serum protein concentration and the relative albumin percentage is an examination that has become employed to a constantly increasing extent in a variety of conditions, as it has been realized how important a part these proteins play in the organism. Not only do such determinations furnish information about numerous pathological conditions, but they also enable us to some extent to assess the state of nutrition of the individual in question. It is, therefore, natural that several investigators have attempted to fix the normal figures of the serum protein concentration and the relative albumin percentage, and the normal variations of these values.

As will appear from Table 1, which shows only the average values, there is a fairly good conformity between the figures stated by the authors mentioned in the table in the case of healthy individuals out of bed in the young, middle-aged and older age groups. As pointed out in particular by LANGE (1946), slightly lower values of total protein are found after only 30 minutes rest in bed. With advancing age, *i. e.* after the fortieth year, there is a slight fall of the albumin percentage in women (BING, NAESER, RASCH and ROWEL, 1946), and after the eightieth year both sexes display a decrease both of the total serum protein concentration and of the relative albumin percentage (Bock, 1948).

Table 1.

*Protein, albumin and relative albumin percentages stated by different investigators.*

Author	n	Sex	Age	Total protein percentage	Albumin percentage	Alb. $\times$ 100	Remarks
						Total	
LINDER, LUNDGAARD VAN SLYKE	8			6.73	4.11	61.3	Oxalat plasma
SALVESEN	14	m		7.07	4.49	63.6	do.
	16	f		7.06	4.33	61.6	do.
PETERS	85	m		6.92	5.03	72.7	do.
EISENMANN	32	f		7.04	5.05	71.9	do.
KUMPF				7.11	4.92	69.5	Serum.
LANGE	60	m	20—37	7.52 (out of bed)			Heparin plasma.
	55	f	18—59	7.38 (out of bed)			do.
	23	m	22—49	6.89 (lying in bed)			do.
	23	f	17—53	6.87 (lying in bed)			do.
BING	38	m	6—67	7.19	4.81	66.8	Serum
NÆSER	49	f	2—67	7.00	4.63	66.0	do.
RØJEL	(87)	m +	2—67	7.08	4.71	66.5	do.
RASCH		f					
BOCK	20	m	60—80	6.63 (lying in bed)	4.22	63.6	Serum
	18	f	60—80	6.64	4.38	65.9	do.

Serum protein determinations of the nature dealt with here are, however, both extremely time-wasting and, in part technically difficult, especially when it is desired to perform determinations in a large number of individuals. Therefore, in such group-examinations it will be advantageous to employ other procedures. For this reason it was decided in the present work to perform the analyses with samples of liquid and dried pooled serum from THE STATE SERUM INSTITUTE. This product results from mixing the sera of a large number of individuals and is used in the production of dried serum for transfusion.

### Material and Technique.

DRIED SERUM is made at THE STATE SERUM INSTITUTE according to the following main principles (MARCUSSEN, 1945): the donors are healthy individuals from twenty to sixty years of age who have vol-

Table 3.

*Protein, albumin and relative albumin percentages of spray-dried serum protein preparations from Dec. 1944 to Dec. 1948, representing 2508 persons.*

Sample No.	Series No.	Protein %	Albumin %	$\frac{A \times 100}{T}$	Dates	Number of Persons	Remarks
1.....	758	49.19	30.46	62.0	11. XII.44	216	Cooled 14 days
2.....	1001	49.20	33.07	67.0	15. XII.44	189	do.
3.....	2322	49.95	34.60	69.2	19. VI.44	108	Frozen before drying.
4.....	3126	49.63	33.35	65.9	15. IX.45	108	do.
5.....	3458	49.51	33.95	68.4	6. X.45	108	do.
6.....	3511	49.71	32.32	65.0	9. X.45	108	do.
7.....	3816	49.72	33.61	67.6	27. X.45	108	do.
8.....	3817	49.58	35.37	71.0	27. X.45	108	do.
9.....	3848	49.18	32.50	66.0	27. X.45	108	do.
10.....	3849	49.62	33.33	67.2	27. X.45	108	do.
11.....	3952	49.16	32.81	66.8	8. XI.45	108	do.
12.....	3987	49.67	33.69	67.4	10. XI.45	108	do.
13.....	4021	49.21	33.03	67.1	15. XI.45	108	do.
14.....	4211	49.47	34.38	69.5	27. XI.45	108	do.
15.....	4675	49.60	32.88	66.3	31. I.46	150	Frozen 14 days before drying
16.....	5022	49.23	32.59	66.2	26. II.46	150	do.
17.....	6149	49.33	34.28	69.5	31. III.46	136	do.
18.....	A	49.19	31.67	64.4	22. X.48	63	do.
19.....	B	49.58	33.37	67.3	11. XI.48	166	do.
20.....	C	50.01	34.85	69.7	15. XI.48	107	do.
21.....	D	49.96	33.97	68.0	3. XII.48	35	do.
		49.51	33.27	67.2		2508	

four different pooled sera representing, respectively, 63, 166, 107 and 35 individuals. The mean serum protein percentage for these 371 persons is then computed to be 7.09. Similar conditions are found in the case of the albumin percentage and the relative albumin percentage, *i. e.* the percentage of albumin comprised in the total protein (GILL, 1928). We can thus take it for granted that, in healthy persons out of bed and being from 20 to 60 years of age, the serum protein percentage, the albumin percentage and the relative albumin percentage in this country for the time being are very close on, respectively, 7.09 per cent., 4.80 per cent., and 67.2 per cent. On comparison with Table 1 it may be seen that these values correspond quite closely to what BING, NAESER RASCH and RØJEL (1946) have found by means of the same analytical method when dealing statistically with a material consisting of 87 single analyses.

It should moreover be noted from Table 2 that the relative albumin percentage in the same pooled sera before and after spraying is much about the same. Spray-drying according to the technique employed at THE STATE SERUM INSTITUTE must thus be considered an extremely lenient process which does not even interfere with the highly labile form in which both the serum albumin and the serum globulins are present (GILL, 1938). This seems to apply also to protein-like substances in serum other than the actual serum proteins (*i. e.* albumin and globulins). Thus the content of procaine-esterase in serum seems to be very little influenced by the spraying process (TERR, 1949).

Table 3 shows the results of the examination of 21 different dried serum preparations from a total of 2,508 persons which were made in the course of the period from December, 1944, to December, 1948. As may be seen, the variation of the total protein content from one preparation to another is only slight. The average relative albumin percentage is 67.2, a value that corresponds rather closely to those stated in Table 1 and 2. The relative albumin percentage does not seem to be subject to seasonal fluctuations either, nor does it appear to have changed in the course of the years covered by the examination. Variations of the total protein concentration cannot, of course, be demonstrated in examinations of this nature comprising only analyses of dried serum.

### Discussion.

The values stated here are, of course, non-contributory to the solution of the question whether, with regard to serum protein percentage and relative albumin percentage, a given individual comes within the normal variation. In other fields, however, examinations of the nature mentioned here may be of more than theoretical interest, for instance when the point is to assess the average serum protein percentage or relative albumin percentage of a population or group of population, or for instance, to estimate possible changes from one year to another, seasonal variations and the like. It is natural that chance variations originating from a single individual for statistical reasons will be eliminated when pooled serum from a sufficiently large population is employed. From this point of view an average serum protein concentration of 7.09 per cent and a relative albumin percentage of 67.2 may be



considered indicative of the normal values for this country for the time being. Moreover, the values may be looked upon as an expression of the average state of nutrition of the 371 individuals investigated.

As already mentioned, the percent of protein in the preparations of dried serum is fairly constant from one preparation to another. Unless deviations from the average values of the serum protein fractions in the population should occur, dried serum accordingly may be considered a very suitable material for biological or biochemical experiments. Dried serum has already successfully been employed for pepsin analyses (HUNT, 1948, HARRESTRUP ANDERSEN and Bock, 1948).

### Summary.

1) Investigations into the serum protein per cent and the relative albumin percentage were performed on four different pools of native serum, representing, respectively, 66, 166, 107 and 35 healthy individuals out of bed from 20 to 60 years of age. An average serum protein concentration of 7.09 and a relative albumin percentage of 67.2 was found. After spray-drying the average relative albumin percentage was 67.5.

2) The figures stated for the native serum, representing in total 371 individuals, may be considered to indicate the normal values in this country for the time being.

3) An investigation of 21 samples of spray-dried sera, representing 2,508 individuals showed no significant seasonal or year variations as to the per cent of protein and the relative albumin percentage. The samples originated from the period Dec. 1944 to Dec. 1948. The average per cent of protein was 49.51 and the average relative albumin percentage was 67.2.

4) Spray-dried serum thus may be considered a very suitable material for biological or biochemical experiments.

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## A Note on an Inhibitory Reflex from the Nose on the Rabbit.

By

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### Introduction.

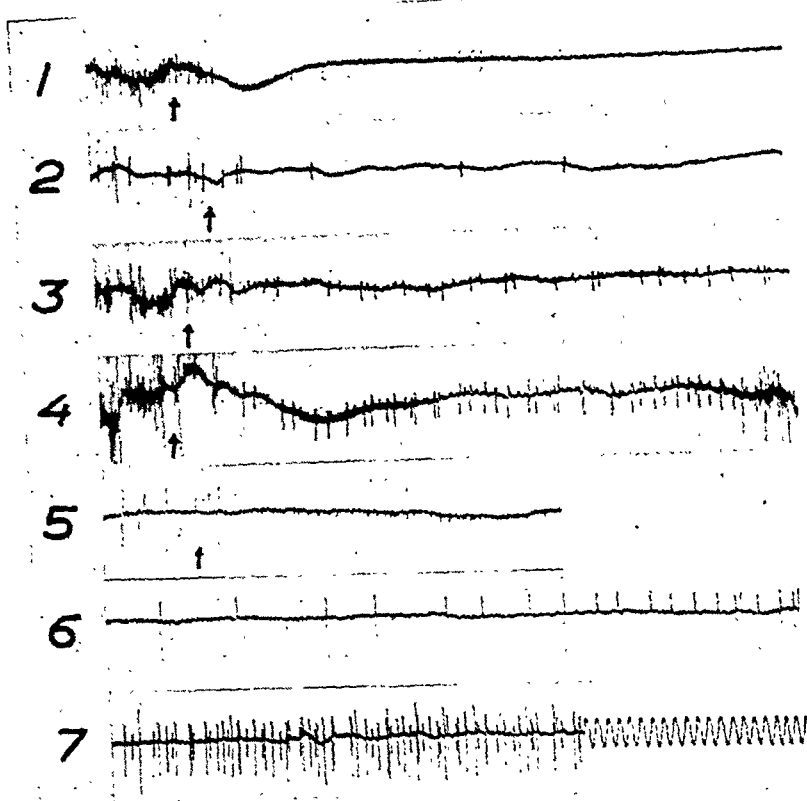
In a previous investigation (FRANKENHAEUSER 1949) it was found that a few drops of ether on the nose of a rabbit in urethane narcosis under certain conditions inhibited motor activity.

The rabbit was completely relaxed and no muscle activity could be registered with an electro-myograph within the first hour of urethane narcosis. After this time "spontaneous activity" occurred in the leg muscles. This activity increased with the time so that it could be seen by the naked eye as twitches in the muscles. The corneal reflex was still abolished. Since this "spontaneous activity" is usually observed when the body temperature of the animal is below normal, it seems likely that the temperature is one of the causal factors. The fact, however, that the phenomenon occasionally appears also when the body temperature is normal indicates that other factors too are operating.

In this investigation the inhibitory influence of ether and smoke on the "spontaneous activity" was studied in eleven rabbits. The same phenomenon was noted in other investigations (FRANKENHAEUSER 1949).

### Technique.

*Narcosis.* A 25 % urethane solution was slowly injected in the marginal ear vein. 1—4 ml. were injected after the corneal reflex was abolished.



The records show the disappearance of motor units when one drop of ether is dropped (marked by an arrow) into the rabbit's nose.

The records are from the following muscles:

1. Quadriceps fem.

2. Platysma.

3. Cricothyreoides.

4. Platysma.

5, 6, 7. Cricothyreoides (same experiment). 6 is taken 8 seconds after 5. Records 6 and 7 are continuous.

Time a. c. 50 cycles.

Further explanation in text.

*Recording.* The motor units were recorded with a double channel resistance condenser coupled differential amplifier. Interference is easier to exclude when a differential amplifier is used (LUNDERVOLD 1947). A modification of the ADRIAN and BRONK (1929) needle electrode was used. The electrode is made of two lacquered copper wires (diameter 0.122 mm.) in an injection needle. The respiration and the time when ether or smoke were given to the rabbit were usually recorded with one channel via a photocell. This particular method of timing is of course approximate since the stimulus does not activate the end organs until it reaches the mucous membrane in the nose. A more accurate method was, however, not needed for these experiments. The impulse activity in the large muscles in the legs and in the respiratory muscles was recorded.

## Observations.

The effect of ether and smoke varied to some extent in the different experiments but in every single experiment, where "spontaneous activity" was observed in the leg muscles, the effect was found to be relatively constant. Experiments on the leg muscles are first described, followed by those on the respiratory muscles.

When a single drop of ether was dropped on the rabbit's nose all "spontaneous activity" was abolished within one tenth of a second (Record 1) in 50 % of the experiments. In 10 % no alteration was observed, in 40 % the "spontaneous activity" was abolished from within one tenth of a second to five seconds (Record 2). In the last mentioned cases most of the units disappeared immediately while some did not disappear until after the next inspiration. Occasionally one or two units remained active (Record 3 and 4). These were usually of small amplitude.

The "spontaneous activity" started again after some time, in some cases already after one second and in some cases after 90 seconds. These are the extreme values which were obtained.

In some experiments an additional drop of ether was dropped on the rabbit's nose every time the "spontaneous activity" reappeared. It was noted that the latent period increased whereas the interval during which no activity was observed decreased successively for each added drop. This continued up to the point when no alteration of the activity was obtained for a new drop.

In many experiments where a few units only were recorded, these were easily distinguished from each other. In some cases the units disappeared within half a second or more. In these experiments it was easy to note the order of both disappearance and reappearance of the single units. It was found that the unit which disappeared first was the last to reappear while the unit which disappeared last reappeared first etc., *i. e.* the order of disappearance and reappearance was reversed. Further it was frequently found that some new units which had not been present before ether, appeared when the inhibitory phase was over (Record 5, 6, 7).

The amount of ether necessary for inhibition is extremely small. Often a single drop of ether 10 cm from the rabbit's nose produced complete cessation of impulse activity. In fact, this circumstance was made the basis of a convenient method of blocking a motor

discharge interfering with the recording of sensory impulses in the rabbit (FRANKENHAEUSER 1949).

The effect of small amounts of ether on some respiratory muscles was also investigated. On auxiliary respiratory muscles, such as the muscles in the larynx, the findings were the same as those described above. In the diaphragm, however, there was never any decrease of the activity.

In some experiments tracheotomy was made. A drop of ether was dropped into the trachea cannula. This did not cause any inhibition of the "spontaneous activity". In some cases the nose was occluded and a drop of ether was dropped into the mouth (the trachea was intact in these experiments). No alteration of the "spontaneous activity" was noted.

A similar inhibition of the motor activity was seen when cigarette smoke was blown on the rabbit. In 10 % of the experiments with ether no inhibition was found. When smoke was used the figure increased to 30—40 %.

### Discussion.

The experiments described show that ether and smoke have an inhibitory influence on "spontaneous motor activity". This cannot be a narcotic affect since the blood concentration of ether is too low, the affect too rapid and, besides, no inhibition occurs when the nose is occluded. It is evident that the irritating agent, ether or smoke, must reach the inner part of the nose in order to be effective. This shows that the inhibition is reflex. The afferent path of the reflex arc must be one of the nerves innervating the mucous membrane of the nose, the olfactory or the trigeminal.

The reason why inhibition failed to occur in some of the experiments remains obscure. Excessive amounts of mucus in the nose (relatively common in rabbits) is a likely explanation.

The adaptation observed might either be a central affair or an adaptation of the end organs. On the basis of these experiments it is impossible to localize the adapting effect.

It is interesting to note that the reverse order of disappearance and reappearance of the motor units during this kind of inhibition corresponds to the recruitments in voluntary movements (SEYFARTH 1941).

### Summary.

Ether and smoke have an inhibitory influence on "spontaneous motor activity" in rabbits in urethane narcosis. The inhibition is due to a reflex elicited in the nose. Adaptation takes place to successive doses of ether. The first inhibited units are the last to reappear after ether.

This work was made possible through a British Council scholarship (B. F.) and a grant from Oslo University (A. L.) for which we wish to express our gratitude.

We wish to thank Doctor GRAHAM WEDDELL for his interest and encouragement in our work.

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## Antihistamine and Respiration.

By

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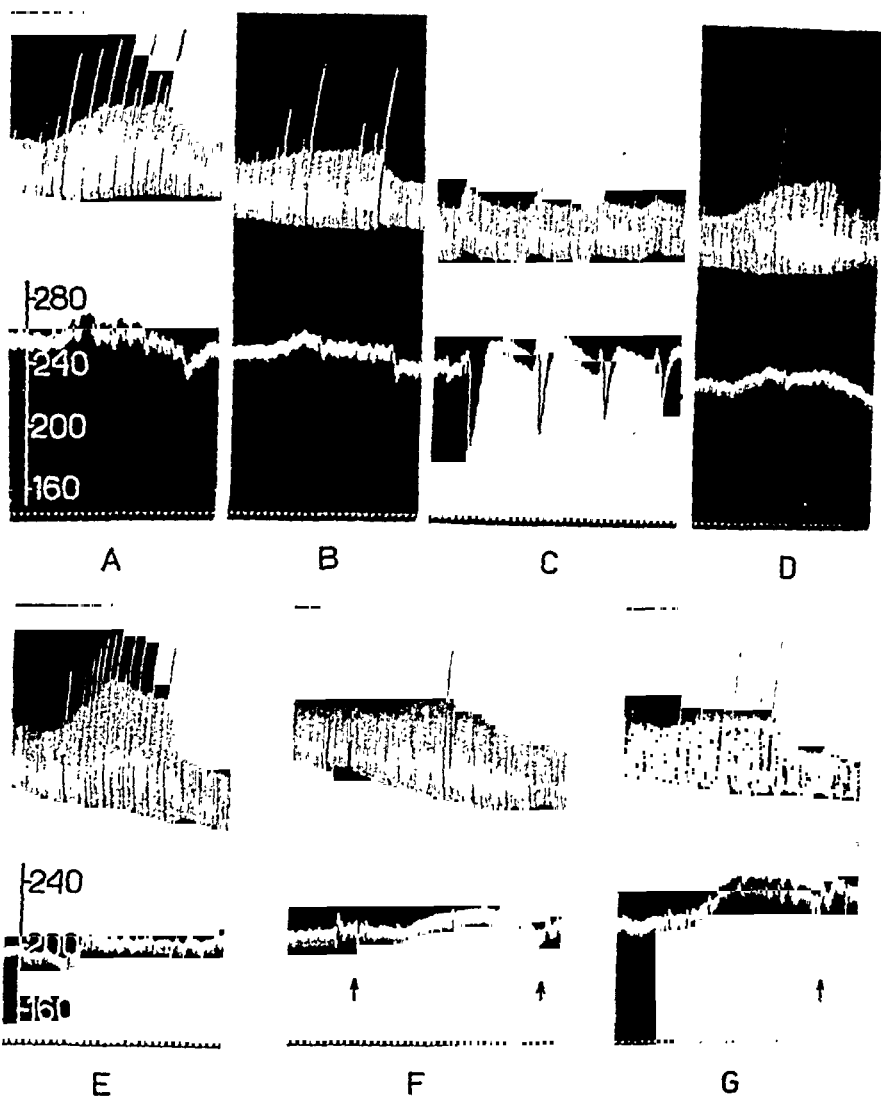
FABINYI and SZEBEHELYI (1948) recently observed that injections of cholesterol into mice prevented the rise in respiratory rate which occurred in untreated animals when inhaling a gas mixture with 10 % of oxygen in nitrogen. The change in respiration due to the inhalation of carbon dioxide mixtures, on the other hand, was uninfluenced. It was further shown in the dog that although neither respiratory rate nor respiratory volume did increase when breathing 10 % oxygen after treatment with cholesterol, the oxygen saturation of the arterial blood was as high as in the animals which had not received cholesterol. But these animals on respiring 10 % oxygen showed a greatly increased ventilation!

According to the authors it seemed probable that the effect of cholesterol was due to its property of neutralizing histamine. Further experiments made by FABINYI and SZEBEHELYI seemed in fact to be in accordance with this hypothesis. After treatment with antistin or desensibilization for histamine inhalation of 10 % oxygen in nitrogen did not increase the rate of respiration as it did before treatment but the increased rate on inhaling 8 % CO<sub>2</sub> in air was uninfluenced. On account of the great theoretical and clinical possibilities that these results seemed to offer the problem was reinvestigated.

### Methods.

Cats were anesthetized with 0,05—0,06 g chloralose per kilogram body-weight. Their respiration was quantitatively measured with the body pletysmograph described by EULER and LILJESTRAND (1936).





Cat weight 2.5 kg. Time marking 10 seconds.

- A. Inhalation of 6.9 %  $O_2$  in  $N_2$  for 3 minutes. Ventilation rises from 570 to 940 ml/min.
- B. Inhalation of 10 %  $O_2$  in  $N_2$  for 3 minutes. Ventilation rises from 590 to 860 ml/min.
- C. Injection of 5  $\mu$ g, 2.5  $\mu$ g, 1  $\mu$ g and 1  $\mu$ g histamine hydrochloride intravenously.
- D. Inhalation of 6.5 %  $CO_2$  in  $O_2$ . Ventilation from 515 to 890 ml/min. Between D and E 13 mg lergitin are injected intravenously.
- E. 6.9 %  $O_2$  as in A. Ventilation rises from 850 to 1365 ml/min.
- F. 6.5 %  $CO_2$  as in D. Ventilation rises from 700 to 1350 ml/min.
- G. 10 %  $O_2$  as in B. Ventilation from 580 to 800 ml/min.

At arrows in F and G injection of 1  $\mu$ g histamine hydrochloride without effect.

The gas mixtures were supplied through the inspiratory Müller valve from bags. The mixtures used were 6.9 % and 10 % oxygen in nitrogen, 6.5 % carbon dioxide in oxygen and 14.9 % carbon dioxide in air. The

blood pressure was recorded with mercury manometer from the femoral artery.

As antihistamine "lergitin" or N-phenyl-N-benzyl-N<sup>1</sup>N<sup>1</sup>-dimethylethylenediamine HCl was used.<sup>1</sup> Solutions of lergitin were given intravenously in doses of 5 mg per kilo body-weight.

## Results.

After inhaling the different gas mixtures for three to four minutes the ventilation had risen to a constant level. Amounts of 0.5—5  $\mu$ g histamine gave satisfactory drops of the blood pressures.

The intravenous injection of 5 mg lergitin per kilo body weight was accompanied by a short period of apnea and a pronounced lowering of the blood pressure as already described (see LOEW, 1947). The period of apnea was followed by a period of hyperventilation, in most cases consisting of increased depth of respiration as well as increased respiratory rate.

After the lergitin injection the histamine doses used gave no blood pressure fall or the drop was greatly diminished. Inhalation of 6.9 or 10 % oxygen, however, still gave a marked increase of ventilation. This effect on the ventilation was at least as large as before the lergitin when the cat inhaled these gas mixtures but sometimes it was not insignificantly greater. The same was the case with the carbon dioxide mixtures. In other words the cats after lergitin showed undiminished respiratory responses when inhaling gas mixtures with low oxygen or high carbon dioxide concentration. Typical results from one experiment are shown in the figure.

## Discussion.

With the quantitative technique used in these experiments no change could be observed in the respiratory adaptation to oxygen lack of the cat after suppressing the action of histamine on the blood pressure. This is in contrast to the results of FABINYI and SZEBEHELYI on dogs. Differences of course may occur between different animals but it is evident that the results of these authors can not be taken as examples of a general principle. It seems also evident that no clinical use may be expected from their finding.

<sup>1</sup> The author is indebted to AB Recip for supplying the lergitin.

### Summary.

Cats were treated with doses of lergitin intravenously that suppressed the blood pressure action of moderate histamine doses. The responses of respiration to inhalation of gas mixtures poor in oxygen or rich in carbon dioxide were undiminished.

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## Occurrence and Metabolism of Free Amino Acids during Insect Metamorphosis.

By

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Only fragmentary reports exist of the nature of the free amino acids which occur in the body fluids of insects. The following authors have dealt with the subject: ACKERMAN (1920 and 1921) — *Melolontha* (Col.), FLORKIN and DUCHATEAU (1942) — *Dytiscus* (Col.), USSING (1946) — *Melolontha* and *Oryctes* (Col.), and RAPER and SHAW (1948) — *Aeschna* (Odon.). According to their investigations and the non qualitative studies of DUVAL and PORTER (1928) — *Attacus*, *Sphinx*, *Cossus* (Lep.), AKAO (1931) — *Bombyx* (Lep.), HELLER (1930 and 1932) — *Deilephila* (Lep.), and FLORKIN (1937) — *Bombyx* (Lep.) the concentration of amino acids in the insect plasma is high.

A qualitative and quantitative establishment of the free amino acids occurring during the insect metamorphosis should be of interest especially for the estimation of the histolytical and histogenetical processes characterizing this period of development. As a link in a continuous study of the intermediate metabolism during the insect metamorphosis the author has attempted, by using the partition paper chromatographic method according to CONSDEN et al. (1944), to follow the variations of free amino acids and related compounds with ninhydrine reaction during the pupal development. This determination is combined with a study of the change in the corresponding hydrogen activating enzyme systems.

## Material and Methods.

The material on which the investigation is based comprises prepupae and pupae in all stages of development of *Calliphora erythrocephala* Meig. (Dipt.) from larvae fed on raw beef and bread at  $+22^{\circ}\text{C}$ , at which temperature the pupal development lasts for about 10 days. Pupae of *Phalera bucephala* L. (Lep.) in diapause as well as in latent and subitan development were also examined, cf. AGRELL (1947).

The following method was employed for the chromatographic determination: Ten *Calliphora*-pupae of the same age were placed into a centrifuge tube where they were crushed and washed with 3.5 ml. distilled water. Protein precipitation with alcohol or  $\text{Ba}(\text{OH})_2 + \text{ZnSO}_4$  according to SOMOGYI (1945) was followed by centrifugation and filtration of the superfluid. Then followed evaporation to dryness in a vacuum exsiccator over phosphorous pentoxide or, in the case of alcohol, distillation. Dissolution was carried out in 0.1 ml. distilled water with slight heating. 2.5  $\mu\text{l}$  of the solution was applied on filter paper (type Munktel No. OB  $50 \times 50$  cm.) for the chromatographic test. The *Phalera*-pupae were treated in a similar manner. Both one- and two-dimensional chromatograms were run at room temperature of approximately  $+18^{\circ}\text{C}$ . The following were used as solvents; pyridine/amylic alcohol (pyridine 35 %, amylic alcohol 35 %, distilled water 30 %) and butyric/iso-valeric acid (n-butyric acid 40 %, iso-valeric acid 40 %, distilled water 20 %). Iso-butyric acid/water was also used as a solvent with good results; on the other hand, even very small amounts of iso-valeric acid, produced lengthy spots, "tails", cf. EDMAN (1945). Unfortunately iso-butyric acid is rather expensive. Altogether some fifty two-dimensional chromatograms were run on the *Calliphora* material as well as three complete series of one-dimensional chromatograms in the alkaline as well as in the acid solvents. For an example of the chromatograms, see Fig. 1. A few samples were studied after acid hydrolysis. Some 10 chromatograms were run on the *Phalera* material; both males and females were used.

The determinations were made on extracts of whole pupae. According to HELLER (1932) the relation between the protein decomposition products in the blood and in the rest of the body is 40 : 60 for *Deilephila* (Lep.). A similar distribution coefficient may be valid for *Calliphora*. It should also be pointed out that amino acids from food in the intestine can hardly influence the results obtained since the intestine is completely voided several days before the formation of the puparium. Moreover, according to ÜSSING (1946), an extensive absorption of amino acids from the intestine content to the haemolymph occurs, as demonstrated on *Melolontha* (Col.).

The chromatographic determinations are supplemented by certain investigations concerning the hydrogen activating capacity of amino acid oxidizing enzymes during the metamorphosis of *Calliphora*. In these experiments the Thunberg-method was used. The change in the rate of the methylene blue reduction, occurring on addition of various amino acids to an untreated pupal mass is described, as well as the cor-

responding Mb-reduction apparent when the pupal substance has been washed free from spontaneous donors and coenzymes in distilled water. In the former case 0.5 ml. Mb in concentrations of 1 : 50,000, 0.5 ml. M/15 phosphate buffer of pH 7.2, and 0.5 ml. of the different amino acids in M/10 were pipetted into every tube. The amino acids used were dl-substances. Alanine determinations also with l-alanine were carried out without any appreciable qualitative and quantitative variation in the results. A pupa was crushed with a glass rod in each tube, which was evacuated for one minute after which the decolorization time was read in a water bath of  $+25^{\circ}\text{C}$ . At the same time control tests were made with the amino acid replaced by a corresponding amount of buffer. The difference in decolorization time was evaluated as a difference in activity according to  $\frac{1}{A} - \frac{1}{B} \times 100$  where A denotes decolorization time after amino acid addition and B decolorization time for the control, cf. AGRELL (1948). The results are given in Fig. 4. Each point of the curves represents the mean value of 10 + 10 tube tests. For determination of the real enzyme activity (in the above-mentioned test spontaneous donors have some influence), three pupae were washed in  $3 \times 10$  ml. distilled water. Following each washing centrifugation at 5,000 R/M was applied and the superfluid discharged. The washed pupal mass was introduced into Thunberg-tubes with the same content as previously stated. Determinations were carried out on l-amino acid oxidase by means of l-alanine and on l-glutamic acid dehydrogenase with this amino acid; in the latter case 0.1 ml. buffer was exchanged for the same amount of 1 ‰ codehydrogenase I solution of about 30 % purity, made according to LEPAGE (1947). Simultaneously a series of control experiments were carried out without amino acid. The results are tabulated in Fig. 5. The activity is evaluated as  $100 \times$  inverse value of decolorization time. Each point represents the mean value of 5 tube tests. The activity of the l-amino acid oxidase is possibly registered as relatively too low because of over dilution.

## Results.

By determinations with partition paper chromatography the following amino acids were demonstrated in the extracts of *Calliphora erythrocephala* pupae (in order of their relative conc.): alanine, glycine, glutaminic acid, valine, proline, leucine, isoleucine, tyrosine, serine, lysine, arginine,  $\beta$ -alanine, aspartic acid, methionine and threonine are found besides the amino sulfonic acid taurine in rather high concentrations and traces of glutamine, two peptides, and one non-hydrolysable compound, probably an amino acid phosphate. The results are presented in the reproduced photograph, Fig. 1. The following amino acids were observed in the extracts of *Phalera bucephala* pupae: lysine, arginine, alanine,

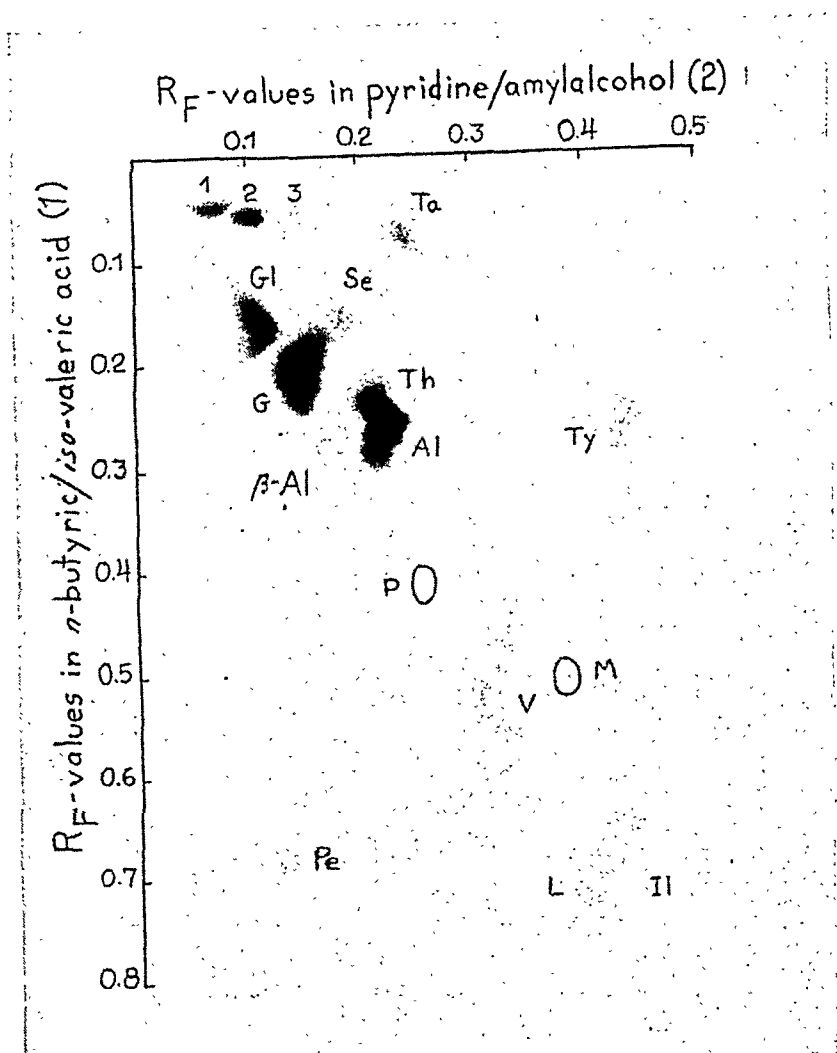


Fig. 2. Photograph of a two-dimensional paper chromatogram using the same extract as in fig. 1. Only are the solvents run in the order *n*-butyric/iso-valeric acid (1), pyridine/amylalcohol (2). Denotations as in fig. 1. 1, 2, and 3 correspond to Ly, Ar and Asp in this fig. Compare also the text.

between the species under investigation is essentially quantitative. The concentration of lysine and arginine is thus much higher in *Phalera* than in *Calliphora* pupae while the concentration of glutaminic acid and tyrosine is comparatively lower. Among earlier investigators ACKERMAN (1920 and 1921) found leucine, lysine, and arginine in extracts of *Melolontha*, but negative result for tyrosine. FLORKIN and DUCHATEAU (1942) reported histidine and tyrosine in the haemolymph of *Dytiscus* but negative results for phenylalanine, arginine, tryptophane, cystine, and cysteine. Ac-

cording to USSING (1946) the bulk of the amino acids in the haemolymph of *Melolontha* consists of lysine, arginine, histidine, leucine, valine, tyrosine, hydroxy-proline, and tryptophane. The presence of alanine, serine, and proline is questioned. However, these determinations are carried out with precipitation and absorption methods, which must be considered less reliable and hardly suitable for determinations on insect material. A comparison with the study of RAPER and SHAW (1948) on the haemolymph of dragon-fly nymphs who also used the partition paper chromatographic method shows closer agreement. In this material: alanine, glycine, valine, leucine, proline, tyrosine, serine, lysine, and arginine were observed, with concentrations decreasing in that order. These amino acids have thus been observed to exist in a free state in three different insect orders, *i. e.*, in Diptera, Lepidoptera, and Odonata.

The chromatograms can be evaluated quantitatively with some certainty. Thus the maximal concentration of any amino acid in both the examined species is about 200 mg %. The interrelationship of the various amino acids, however, is not easily estimated on the basis of intensity and size of the spots. On comparison, different amino acids give nin-hydrine reaction of varying strength, *cf.*, for instance, DENT (1948), and sometimes also varying size of the spots although the same concentration was used. But the amount of the same amino at different developmental stages can be compared, and an eventual fluctuation in amino acid concentration during the metamorphosis period established. As was previously mentioned both one- and two-dimensional chromatograms were run. For the sake of simplicity however the amino acid concentration during the pupal development of *Calliphora* is represented by two series of one-dimensional chromatograms, in pyridine/amylalcohol and in n-butyric/iso-valeric acid respectively, Fig. 3. Repeated experimental series made on different occasions produced identical results. The only slight variation in the concentration is particularly striking, but agrees with, for instance, HELLER (1939), and FLORKIN (1947). The quantity of primary free amino acids is remarkably constant during the entire metamorphosis period in spite of a succession of profound histolytic and histogenetic processes. There is thus balance between the formation of free amino acids through proteolysis on the one hand and on the other their consumption through oxidation and protein synthesis.



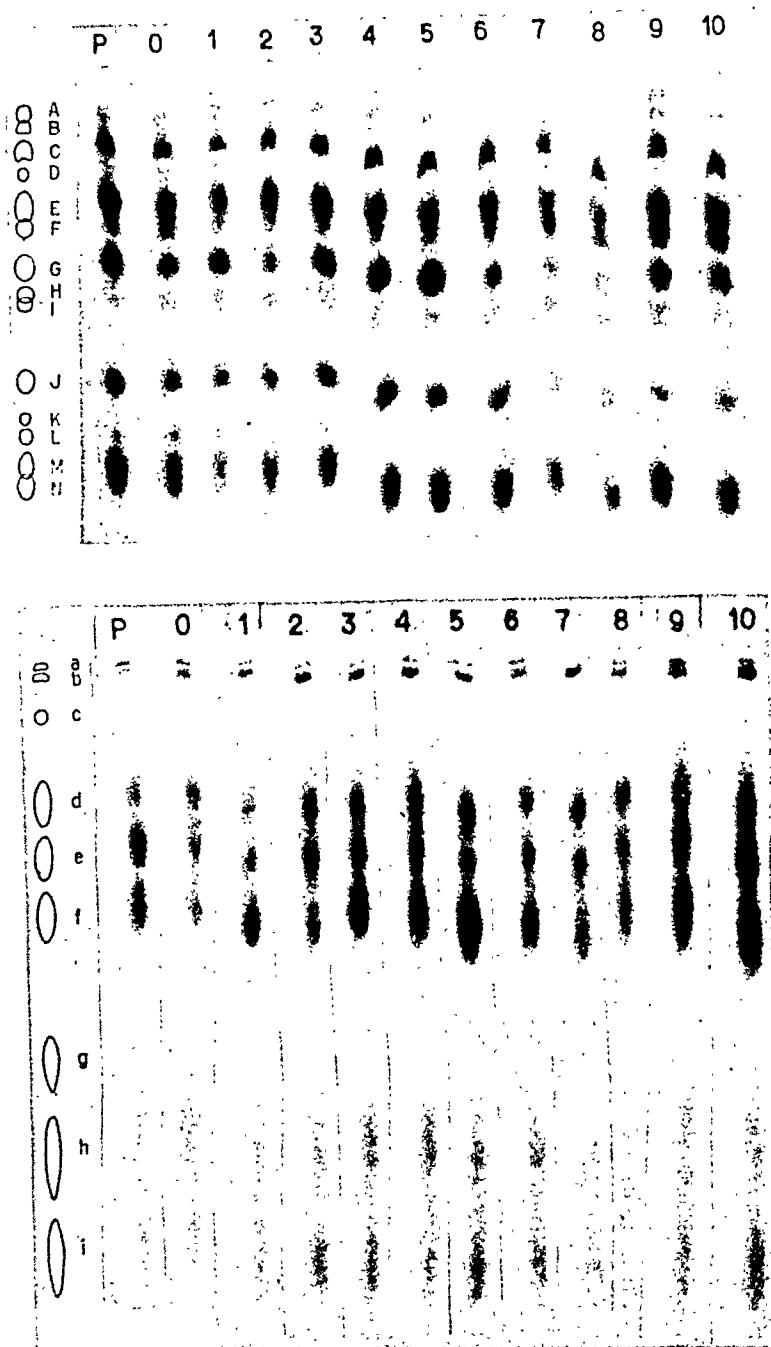


Fig. 3. Photograph of one-dimensional paper chromatograms on extracts from *Calliphora*-pupae in different developmental stages. P prepupa, 0—10 pupal age in days. Upper chromatograms run in pyridine/amylalcohol: A lysine, B arginine, C glutaminic acid and a peptide, D aspartic acid, E glycine, F serine and  $\beta$ -alanine, G alanine, H proline (not visible on the photograph), I taurine, J valine, K methionine, L tyrosine, M leucine, N iso-leucine. Lower chromatogram run in *n*-butyric/iso-valeric acid: a and b lysine, arginine and aspartic acid in phosphorylated (?) state, c taurine, d glutaminic acid and serine, e glycine and threonine, f alanine,  $\beta$ -alanine and tyrosine, g proline (not visible on the photograph), h valine and methionine, i leucine, iso-leucine and a peptide.

cate that the amino acid formation, proteolysis, is in dynamic equilibrium with the amino acid consumption, oxidation and protein synthesis. Thus, either a comparatively increased proteolysis must be assumed in the formation of the puparium and immediately prior to the hatching, which seems fairly improbable, or a comparatively reduced protein synthesis at these points of time, which appears more likely. A relative maximum in proteolysis can instead be assumed to occur in the middle of the metamorphosis at 30–50 % of the pupal time.

Even though the amino acid concentration during the metamorphosis is largely constant a certain variation can be noticed. Thus a slight minimum occurs at 0–20 % pupal time and a more marked minimum at 60–80 %, Fig. 3. It is likely that these temporary fluctuations are connected with the histological changes occurring at these points of time. At 60 per cent of the pupal time there is a rapid increase in muscular protein, the thoracic musculature appears, and this histogenetic process is quantitatively wholly dominant at this period. This protein synthesis is likely to affect the amount of primary free amino acids either directly or through reduction in the proteolysis, *i. e.*, reduced decomposition into amino acids. Either way the amino acid concentration should fall, which is what happens. The amount of total protein also varies in a similar way. At 80–90 % pupal time the muscle formation is quantitatively completed and the amino acid concentration rises to the earlier steady value. The less considerable fluctuation in the amino acid concentration following the formation of the puparium may be associated with the simultaneous histogenesis of the hypodermal tissue. Thus the imaginal buds appear at about the same time as the puparium, they are evaginated at about 10 % of pupal time and at 20–30 % of pupal time the external organization of the imago is in principle finished. The development of the brain occurring mainly at this time might also be a contributory factor.

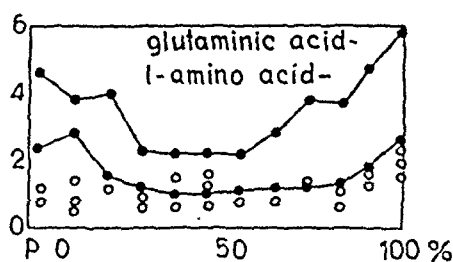


Fig. 5. The variation in activity of l-glutaminic acid dehydrogenase, upper curve, and of l-amino acid oxidase, lower curve, during pupal development of *Calliphora*. Unfilled circles represent controls with only codehydrogenase I added. Vertical axis: rate of Mb-reduction. Horizontal axis: pupal age in %.

All free amino acids are not to the same degree affected by the abovementioned fluctuations in concentration, Fig. 3. The concentration of glutaminic acid, glycine, proline, valine, leucine, isoleucine, and also taurine varies to a lesser extent. Alanine exhibits the same change in concentration though more pronounced — a maximum of about 100 mg. % and a minimum of about 25 mg. %. This reduction in concentration may be the cause of the increased Mb-reduction on addition of alanine to the pupal mass at approximately 70 per cent of pupal time, Fig. 4. The concentration of  $\beta$ -alanine increases towards the end of the pupal period. Threonine and serine show irregular variations. Methionine has only been demonstrated at the beginning and at the end of the metamorphosis period. The concentration of lysine, arginine, and also aspartic acid is essentially the same at every point of time examined. A certain peculiarity in the behaviour of the last-mentioned substances in the chromatograms should be pointed out. If two-dimensional chromatograms are run so that pyridine amyl-alcohol is first used as solvent followed by n-butyric/iso-valeric acid, the  $R_F$ -values for lysine, arginine, and aspartic acid conform with those of corresponding test substances, Fig. 1. The same occurs if phenole (1)/collidine (2) is used. If, on the other hand, the chromatograms are run in the order n-butyric/iso-valeric acid (1), and pyridine/amylalcohol (2) the  $R_F$ -values in pyridine/amyl-alcohol become approximately comparable to those of the test substances while extremely low and almost identical  $R_F$ -values are obtained in n-butyric/iso-valeric acid, Fig. 2. The same thing happens in one-dimensional chromatograms with the last-mentioned solvent only, Fig. 3. Apparently arginine, lysine, and aspartic acid are present in the pupae in combination with some other substance, as, for instance, phosphate, the labile bounds of which are easily split by pyridine and also by phenole. The same seems to be valid for their occurrence in *Phalera*-pupae. A similar phenomenon has been observed with respect to glutaminic acid. As was previously pointed out, two protein precipitating substances were used, alcohol and  $\text{Ba(OH)}_2 + \text{ZnSO}_4$  according to SOMOGYI. The Somogyi-solution precipitates glutaminic acid and also aspartic acid almost completely. In spite of this, the quantity of glutaminic acid obtained in the chromatograms is always the same, and is independent of the precipitation substances and solvents used. This indicates that also native glutaminic acid exists not as free glutaminic acid but as a constituent of another com-

pound which is not precipitated by Somogyi-solution and is split up under the influence of the solvents in question. This theory is further substantiated by the fact that an addition of glutaminic acid to the untreated pupal mass produces an increase in the Mb-reduction, which during the metamorphosis varies in a way, Fig. 4, hardly concordant with the variation in the glutaminic acid dehydrogenase activity, Fig. 5, if that is assumed, which the chromatograms should prove, that the real concentration of glutaminic acid changes only little during the metamorphosis period.

A quantitative evaluation of the chromatographic results from extracts of *Phalera* pupae gives apparently divergent results. The pupae were examined with regard to free amino acids during the diapause and the latent and subitan developmental periods, cf. AGRELL 1947. During the diapause the total metabolism is at its minimum; during the latent period the developmental potency increases almost without any corresponding increase in the metabolism; during the subitan development the metabolism increases markedly and the animals hatch. The concentration of free amino acids increases during the latent period but decreases obviously during the subitan development. This is valid for both males and females. The difference to conditions in *Calliphora*, however, is probably only apparent. During the subitan development the main formation of the imaginal musculature occurs. This period of the histogenesis is also characterized in *Calliphora* by a reduced concentration of free amino acids. It is likely, therefore, that if determinations of the amount of amino acid had been carried out somewhat later during the subitan development of *Phalera* pupae an increase in concentration would have been observed.

Valuable technical work has been carried out by Miss ELSA ROSENGREN. For stimulating discussions and his kindness in lending me chromatographic apparatus I am indebted to Dr. LENNART JACOBSSON. Mr. RUNE STJERNHOLM has been kind enough to check my results by chromatography in phenole-collidine for which I thank him. The investigations were carried out with the financial aid of the Scandinavian Insulin Foundation and Swedish Natural Science Research Council.

### Summary.

1. With the partition paper chromatographic method the occurrence of free amino acids in extracts from pupae of *Calliphora*

*erythrocephala* MEIG. (Dipt.) and *Phalera bucephala* L. (Lep.) were investigated. For both the species principally the same amino acids were found.

2. The amino acid concentration in *Calliphora* is comparatively constant during the whole development. There are, however, slight minima at 0—20 % and 60—80 % of pupal time. These minima may be put in relation to the histogenesis of hypodermal and muscular tissue, respectively. The *Phalera* pupae show an increased amino acid concentration during the latent development and a decrease during the subitan development.

3. By the use of Thunberg's methylene blue method it was found that during the metamorphosis of *Calliphora* the enzymatic amino acid oxidation has a maximum activity at the beginning and at the end of the pupal period but is at its minimum in the middle of this period in accordance with the total metabolism. The interrelationship between amino acid oxidation, protein synthesis, and proteolysis is discussed and it is suggested that a minimum protein synthesis exists at the formation of the puparium and at the end of the pupal period, a maximum protein synthesis at pupation and during the histogenesis of the imaginal muscles after the middle of the metamorphosis period, and a relative maximum of proteolysis at 30—50 % of the pupal time.

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## A Method for Reducing the Variation in Metabolism Tests, with Application to the Effects of Temperature, Sex and Weight on the Oxygen Consumption in Guinea-Pigs.

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Let us suppose that we are to ascertain the effects of drugs on the metabolism, it will be found that the effects rarely exceed  $\pm 30\%$  of the basal metabolism if we take the average of several tests. The individual variation in such tests depends on the drug examined, on the kind of animal subjected to the test, and, to some extent, on the method of procedure. The variation in effect is made up of two components, firstly the variation in the basal metabolism, and secondly a variation due to different sensitivity to the drug in question. The total variation is thus never less than that of the basal metabolism. A study of the variation in the basal metabolism is therefore of interest as indicating the minimum variation that may be expected in testing the effect of drugs on the metabolism.

In Table 1 the figures given in the literature for the variation in the basal metabolism in man and the usual laboratory animals are compared. The gauge taken is the coefficient of variation, which indicates the standard deviation in percentage of the mean ( $\frac{\text{standard deviation} \times 100}{\text{mean}}$ ). The coefficient of variation in cases

where it has not been stated has been computed on the basis of the information given by the respective author. The variation

Table 1.

*Variation of basal metabolism in the same individual on different occasions and between different individuals, in man and animals. The variation is shown as the standard deviation in percentage of the mean = the coefficient of variation. The body surface of the animals is estimated according to Vierordt's formula, where K (for cat and rabbit) = 10. Dog 11.2, rat 9.1, guinea pig 8.0.*

Species	Body weight range kg	Cal/m <sup>2</sup> per day	Coefficient of variation		No. of observations	No. of individuals	Author
			within individuals	between individuals			
Man ....	57—73 —	902 925	4 —	— 7	364 —	11 136	HARRIS and BENEDICT (1921)
Dog ....	12.8—17.5	704	4.5	—	26	3	BOOTHBY and SANDIFORD (1923)
	8.8—26.8	771	—	7	—	27	KUNDE and STEINHAUS (1926)
Rabbit..	— 1.15—7.0	— 684	8 —	— 14	96 —	8 74	LEE (1939)
Rat ....	0.054—0.66	712	8	22	130	41	HORST, MENDEL and BENEDICT (1934 a, b)
	0.108—0.163	1096	—	11	—	45	KRANTZ and CARR (1942)
Guinea pig ....	0.440—0.650	1003	11	16	74	12	LUNDHOLM and MOHME (1949)
Cat ....	1.35—3.30	728	13	23	37	14	LUNDHOLM and MOHME (1949)

in the basal metabolism is tabulated 1) as a variation in the same individual on different occasions (days) and 2) as a variation between different individuals. The coefficient of variation first-indicated is of particular interest in studying the effect of a drug on the metabolic rate, seeing that the effect thereof on the metabolism is usually compared with the basal metabolism of the same individual.

The figures given for individual variations are, of course, merely approximative, as they partly depend upon the length of time

for which the observations have been carried on. The coefficient of variation in fact increases according as the observation period is extended (HARRIS and BENEDICT, 1921). As the length of the observation period differs considerably in different cases (a fortnight—2 years), the comparisons should be made with some caution. It is evident, however, that the smallest individual variations are shown by man and by dogs, which is partly due to the fact that they can be trained in respiratory tests. Rats, rabbits and guinea pigs show much the same individual variation, whereas the figure is considerably higher for cats.

The variation *between* individuals depends on several factors, such as age, weight and sex, as is clearly shown by the observations on the rat. Thus, for rats varying in weight between 54 and 660 grams the variation amounted to 22 %, whereas it fell to 11 % if the range of weight was 108 to 163 grams. Hence the figures for the coefficient of variation between individuals are still more approximative than those for variation within the same individual. As indicated by the Table, however, the variation between different individuals is in all cases considerably greater than that within individuals, which shows the desirability of comparing the effect of a drug on the metabolism with the same individual's basal metabolism.

If we are to ascertain if there is a block between two drugs in regard to their effects on the metabolism it must be shown with statistical validity ( $P < 0.01$ ) that  $A + B - AB > 0$ , where A is the effect of the drug blocked, B the effect of the blocking drug and AB the simultaneous effect of the two substances on the metabolism. Let us suppose that in tests on guinea-pigs  $A = +15\%$  of the basal metabolism, that  $B = +5\%$ , and that B completely blocks A. The smallest number of tests required in order to show the blocking effect with the stipulated significance can then easily be computed. The standard deviation in A, B and AB is at least 10 %. If the same number of tests (N) of A, B and AB are made, we obtain, ac-

cording to statistical rules:  $15 + 5 - 5 = 15 \pm \sqrt{\frac{10^2}{N} + \frac{10^2}{N} + \frac{10^2}{N}}$ .

In order to obtain  $P \leq 0.01$ ,  $\frac{15}{\sqrt{\frac{300}{N}}}$  must be  $\geq 2.5$ , that is to say,  $N \geq 8.3$ . Thus, in all, at least 27 tests must be made in order to



show with statistical significance that B blocks A. The figure of 10 % for the standard deviation, however, is a minimum, so that sometimes we must reckon with 15 %, in which case the total number of tests will amount to 57. If, besides this, the blocking is not complete, but merely as to 50 %, 224 tests will be required.

These examples indicate that it is rather hazardous to attempt, without very cogent reasons, to show blocking between drugs on the metabolism. In testing such effects, it is therefore highly desirable to try and reduce the variability. One alternative is as indicated by Table 1 to make the tests on man or dogs. The attempted reduction, however, will be rather moderate, as the variation due to the action of the drug will persist. Thus, in 46 tests with adrenalin 10  $\gamma$ /kg on man the coefficient of variation was 8.8 % (SANDIFORD, 1920) whereas the corresponding variation in 39 tests with 20  $\gamma$  adrenalin per kg on guinea pigs was 11.3 % (LUNDHOLM and MOHME, *op. cit.*). It seems therefore that the advantage in the form of reduced variability, when weighed against the greater technical difficulties presented by man and dogs compared with the smaller animals, is not very great.

Another alternative is to make several tests with the drug on the same animal, in the expectation that the effect will then be more uniform. This, however, is not always the case. Thus, in tests with adrenalin, the variations shown by an individual guinea pig were quite as marked as the dissimilarities in this respect observed in tests on different guinea pigs (LUNDHOLM and MOHME, *op. cit.*). From this it follows that to select animals of the same litter, weight, sex or age in order to reduce the variations will not always lead to successful results.

It may be inferred from the above that, in testing the effect of drugs on the metabolism, there is at present little prospect of reducing the variations. On the other hand, the labour involved in conducting a large number of tests can be somewhat reduced. This can be done by conducting several tests simultaneously, *e. g.* in one of the apparatuses with several compartments described by BENEDICT (1930) or KIBLER and BRODY (1942). This will serve, in a moderate degree, as a labour-saving device.

A considerably greater economy of labour, in conjunction with certain other advantages can, however, be effected by the method which will now be described. It is based on the procedure that several animals are introduced into the same respiration chamber, whereupon their total respiratory exchange is measured. This

method is by no means new, having been used as far back as 1895 by SONDÉN and TIGERSTEDT and subsequently on several occasions by BENEDICT and co-workers (BENEDICT and MACLEOD 1929, BENEDICT and RITZMAN 1931, BENEDICT and FOX 1933). It should be noted, however, that what induced the said authors to test several individuals simultaneously was the desire to increase the output of carbon dioxide, so that it could be measured with greater accuracy. The only investigators who seem to have availed themselves of the procedure in order to reduce the variation are KROGH and LINDBERG (1931), who, however, did not consider themselves to have attained the desired effect.

The chief advantage of such a method is that the results obtained by simultaneously testing several animals show less dispersion than those based merely on a single animal. If the observations on single animals are distributed according to a curve with the mean  $M$  and the standard deviation  $\sigma$  the sample of means which are based on the random choice of  $a$  variate from this population have the same mean  $M$  but the standard deviation  $\frac{\sigma}{\sqrt{a}}$ . But even if the observations on single animals are distributed far from normally the sampling distribution of the means approaches more and more the normal form as characterized by skewness and kurtosis as the value of  $a$  increases. (KENNEY 1939 II. s. 111.) This may be advantageous in certain cases, the mathematical laws derived from the normal curve have stricter validity when the observations are based on several animals than when they are based on single animals. But it has certain drawbacks as the distribution of the tests on several animals does not admit of any reliable conclusions being drawn in regard to the distribution of the tests on single animals.

The saving of labour entailed if the tests are made on several animals simultaneously, instead of on single animal separately, may be expressed by the ratio  $\frac{N}{n}$ , where  $N$  is the number of tests on single animals required in order to attain a certain degree of statistical validity, and  $n$  the number of tests on several animals simultaneously that may be required for the same purpose. If  $N$  and  $n$  are numbers large enough for the strict application of the regular statistical rules,  $n$  will be  $= \frac{N}{a}$ ,  $a$  representing the number of animals simultaneously tested. The purpose of the method, however, is to reduce  $n$  (the number of tests) so far as possible,

regardless of the sign, will not exceed  $t$ . Thus,  $P = 0.01$  according to STUDENT corresponds, according to FISHER, to  $P = 0.02$ . In Table 2 the last-mentioned method has been followed. The table indicates the number of tests required in order to show with different degrees of validity that a percentage deviation is greater than 0.

As indicated by Table 2, the economy of labour effected by making the tests on several animals simultaneously will be greater the larger the number of tests on single animals that would have been required in order to attain a certain degree of validity. If the coefficient of variation is small, not much will be gained by making tests, say, on 9 animals instead of 4 simultaneously. But, if the coefficient of variation is large, it will be advantageous to make tests on as many animals as possible. Seeing that as a rule the coefficient of variation is not known in advance, it would be best, at any rate at the commencement of a series, to confine oneself provisionally to tests on 4 animals and on this basis to increase or reduce the number of animals.

In the analysis of variance, the conditions are more complicated. In endeavouring to ascertain whether the variation *between* a number of groups is significantly larger than that *within* the groups, the minimum number of tests that must be made is, theoretically, equivalent to the number of groups + 1. In practice, however, it may be considered necessary to make at least two tests per group, the result being that, if the number of groups is large, a considerable number of tests will have to be made in any case. The implication is that the additional saving of labour effected by testing more than four animals will as a rule be rather insignificant.

From another point of view, however, it may be advantageous to test a larger number of animals simultaneously. If, in a certain test, the number of groups, owing to the nature of the problem, cannot be increased and the ratio between the variances is small, it will scarcely be possible to show satisfactorily by variance analysis any marked difference between them. If, however, the tests are made on  $a$  animals simultaneously, the variance within the groups will be reduced  $a$  times, and it may then be possible to show statistically significant differences. The above can be easily deduced from the tables for variance ratios.

The method described above has been elaborated in order to make it possible with a reasonable amount of labour to investigate

the inhibiting and potentiating effects of drugs on the metabolism. It is adapted also for ascertaining the relation between the dosage of a drug and its said effect. As a rule, in fact, considerable variations in the dosages of a drug are required in order to show distinct differences in its effect on the metabolism. Should the variation in effect be reduced, it may be possible to detect even minor differences.

As previously pointed out, there is also a considerable variation in the basal metabolism of different individuals. By combining groups of animals of the same sex, weight and age, it will be possible to facilitate the study of the effect which those factors have on the basal metabolism. The variation that had been viewed as an "error" will be reduced by  $\frac{1}{\sqrt{n}}$ , whilst the effect produced by one of the said factors will remain unchanged.

In the following it will be shown by a few examples that the distribution of a series of tests (1) with a single animal and (2) with 4 animals in the group will fairly well follow the mathematical laws previously mentioned. The results of an investigation into the effect of temperature, weight and sex on the basal consumption of oxygen in guinea pigs will moreover be reported.

### Methods.

The tests were made on guinea pigs, four in each group, with a closed circuit apparatus previously described (LUNDHOLM and MOHME *op. cit.*). The oxygen consumption was recorded, in periods of 5 minutes, on a kymograph for one hour. Before the tests, the animals had been kept without food for 16—22 hours. They received fodder consisting of hay, turnips and oats, besides fresh grass during the summer months. The tests were made (1) at 26° C and (2) at 32° C, the figures indicating the temperature of the water bath. In cases where the tests were made at a temperature of 26°, the animals had previously been kept at a temperature of 20—22° C. When the tests were made at 32° the animals were kept for 1—2 hours at that temperature before recording the oxygen consumption.

When the animals had been put in the respiration chamber, which had a capacity of 10 litres and was divided by metal network into 4 compartments, the oxygen consumption was recorded after the lapse of 10 minutes. In some cases the animals had received a subcutaneous injection of 1 cc. 0.9 % NaCl before being placed in the respiration chamber. This, however, did not affect the oxygen consumption, as the effect of the injection had passed off after 10 minutes (LUNDHOLM and MOHME, *op. cit.*). At first the activity of the animals was recorded with an air cushion connected with a Marey's capsule. But, as the

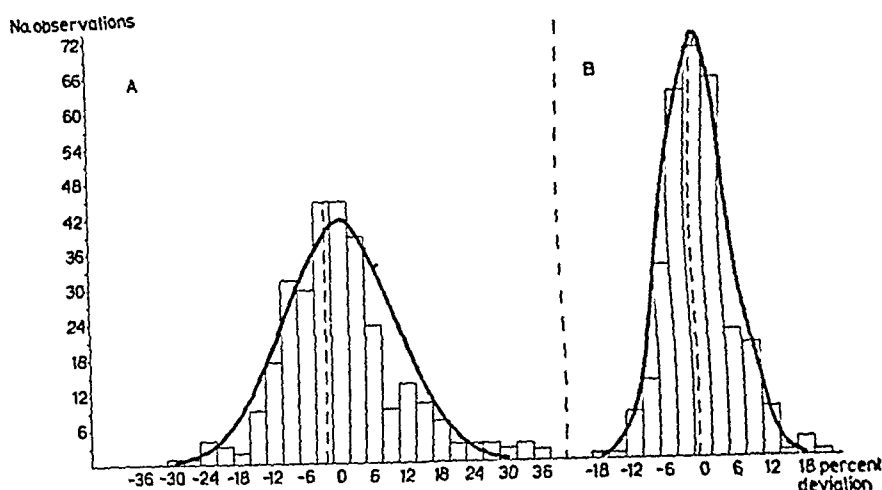


Fig. 1. Histogram for the oxygen consumption during 300 periods of 5 minutes. Abscissa shows deviation from mean in percentage. Class range 3 %. Ordinate shows number of variates (class frequencies). The median and the calculated normal curves are inserted in the graph. A. observations on single animals. B. observations on groups of 4 animals simultaneously.

animals, generally speaking, were very quiet, this procedure was soon abandoned.

The surface area of the body was measured as regards 16 animals who had been kept without food for 22–24 hours. When they had been weighed they were killed with amytal and inelastic threads were securely sewn in the skin in the following way: A longitudinally running thread from the tip of the nose to a position just dorsally of the anus. Three circular sutures, one round the narrowest part of the neck, one round the sternum on a level with the ensiform process, and one round the abdomen on a level with the proximal part of the os sacrum. The threads were then tightened, though not tensely enough to prevent the animal from apparently lying in a natural position. The skin was then loosened and drawn taut enough to stretch the threads. The skin over the extremities, where there were no stitches, was moderately tightened. The contours of the skin were traced on paper, the resulting figure pattern being then cut out and weighed. The surface area of the skin was then computed on the basis of the weight of the cut-out paper and the weight of 100 square cm of the same paper.

## Results.

Fig. 1 shows the distribution curve for 300 periods of five minutes firstly in tests on a single animal (A) and secondly in tests on 4 animals simultaneously (B).

The values were obtained by first calculating the average oxygen consumption for 5 minutes for the last 30 minutes of a test; the percentage deviations from this value for each of the six periods

of 5 minutes were then computed. This was done in 50 tests, each of which was made on a single animal and on 4 animals. The values for a single animal had been taken from a previous work (LUNDHOLM and MOHME *op. cit.*). The estimated normal curve as well as the median have also been inserted in the figures. In the tests on single animals the standard deviation and its standard error were  $10.1 \% \pm 0.41$  and the range of variation  $-31 \%$  to  $+37 \%$ . In the tests on four animals the corresponding figures were  $5.66 \% \pm 0.23$  and  $-17 \%$  to  $+21 \%$ . The standard deviation, in conformity with the theory, had thus been reduced approximately to half. It may be expected a priori that the distribution curve for single animals will be somewhat skew, as periods of activity produce high values which are not set off by any low ones. This was in fact actually the case, as shown by the value of the median,  $-2.55 \%$ . In the tests with 4 animals the value of the median was  $-0.57 \%$  and the skewness had thus been reduced. In order to obtain an exact numerical value for the skewness, it was thought necessary to compute the ratio skewness =

$$= \frac{\sqrt{\beta_1} (\beta_2 + 3)}{2 (5 \beta_2 - 6 \beta_1 - 9)}$$
 where  $\beta_1 = \frac{\mu_3^2}{\mu_2^3}$  and  $\beta_2 = \frac{\mu_4}{\mu_2^2}$ , where  $\mu_2, \mu_3, \mu_4$  are the second, third and fourth moment about the mean (YULE and KENDALL 1947, p. 162). For a normal curve this ratio is 0. For curve A the figure was 0.240 and for curve B 0.128. We thus see that in this case too the experimental result corresponds with the theory.

That this applies also to tests of the effect of drugs on the metabolism is shown by curve A in Fig. 2, being the distribution curve for the effect of 20  $\gamma$  adrenalin per kg in 39 tests on single animals. The values indicate the percentage increase of the oxygen consumption 10–100 minutes after a subcutaneous injection of adrenalin, relatively to the basal oxygen consumption measured for 60 minutes before the injection. (See LUNDHOLM and MOHME, *op. cit.*, from which the values are taken.) The standard deviation was  $11.3 \pm 1.0$  and the range of variation  $-9.5 \%$  to  $+37 \%$ , the mean  $+12.6 \%$  and skewness = 0.90. In 22 tests on four animals simultaneously with the same technique, the standard deviation was  $7.2 \pm 0.94$ , the range of variation  $-2.5 \%$  to  $+19.5 \%$ , the mean  $+8.8 \%$ , the skewness = 0.42.

In view of the small number of tests, the correspondence between the theory and the experimental result must be considered to be rather good.

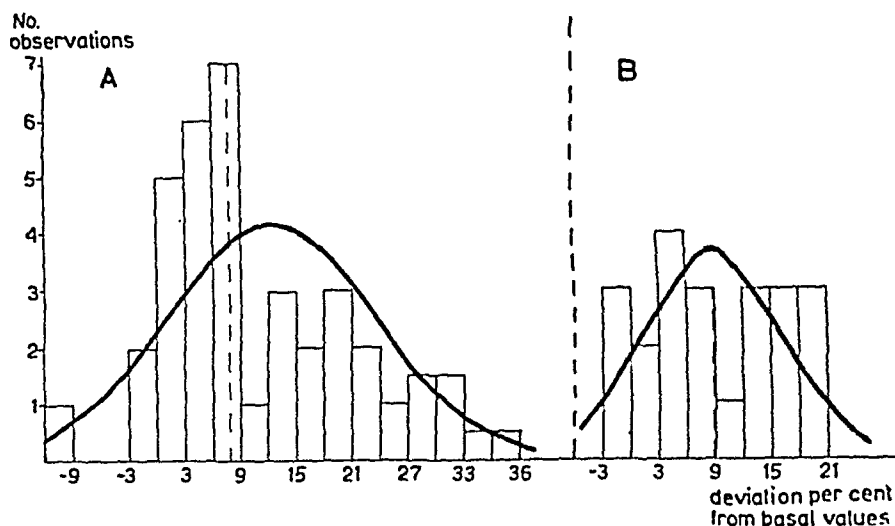


Fig. 2. Histogram showing effect of 20  $\gamma$  adrenalin per kg on oxygen consumption of guinea-pigs. Abscissa shows in percentage the change in oxygen consumption compared with basal consumption. Class range 3 %; Ordinate: number of observations. Median and calculated normal curves inserted in the graph. A. observations on single animals. B. observations on groups of 4 animals simultaneously.

The effect of sex, weight and temperature on the oxygen consumption was investigated on guinea-pigs, in groups of four, composed so as to secure uniformity in regard to sex and weight. The maximum permissible difference in weight within a group was 200 grams. As the age of the animals was not exactly known in all cases, we refrained from considering the effect of that factor on the oxygen consumption. The age, however, can be approximately estimated from the weight ; thus, a weight of 300 grams corresponds to an age of ca. 2 months, 400 grams to 4 months, 800 grams to 12 months. In all, 154 tests were made on 25 different groups of four. The total number, however, somewhat exceeded a hundred, being 116, viz. 53 males and 63 females. In fact, when one of the animals, as happened in some cases, had fallen ill or died, it had to be replaced by another of the same sex and weight.

The results of these tests may be regarded as fairly representative of the average effect of sex, weight and temperature on guinea-pigs weighing from 250 to 1,100 grams.

It should be pointed out that, if the effect of a certain factor on the metabolism is to be studied and if the same animals cannot be used for the purpose, a relatively small number of tests should be made on many different animals of the same species, and not a

Table 3.

*Composition of the material, and other statistical data. The body surface computed according to the experimentally found formulae for males and females.*

	Temperature 26° C		Temperature 32° C	
	Males	Females	Males	Females
No. of observations...	38	42	39	35
No. of groups .....	6	6	5	8
Mean of the weight of the groups in gm and its standard deviation .....	2352 ± 818	1962 ± 762	1959 ± 410	2868 ± 651
Weight of the groups, range in gm .....	1260—3740	1140—3340	1395—2860	1560—4100
Weight of individual animals, range in gm	290—1160	250—890	300—840	340—1100
Mean of the oxygen consumption cc/m <sup>2</sup> per min. and its standard deviation .	130.5 ± 11.8	131.2 ± 8.7	118.5 ± 6.4	114.1 ± 7.4
Oxygen consumption cc/m <sup>2</sup> per min. range	115—157	117—154	106—139	102—139
Oxygen consumption cc/m <sup>2</sup> per min. calculated at 500 gm body weight .....	133.3	131.0	118.4	119.4
Cal/m <sup>2</sup> per day at 500 gm body weight. 1 liter O <sub>2</sub> = 4.7 Cal...	902	887	801	808

large number of tests on a few animals. In view of the marked variation between individuals, differences between them can easily be shown; these differences, however, should not necessarily be attributed to the factor that is being temporarily investigated.

A general survey of the material, arranged according to temperature and sex, is given in Table 3. The several tests are shown in Fig. 3. The oxygen consumption is indicated in O<sub>2</sub> cubic centimetres per minute per sq. metre of the body surface.

The area of the body surface was first computed according to Vierordt's formula: body surface =  $K \cdot \text{weight}^{\frac{2}{3}}$ ; ( $S = K \cdot W^{\frac{2}{3}}$ ) ( $K = 8.0$ ). After computing the surface area of each separate animal, the surface area of the entire group was calculated as the sum-total of those figures. It appeared from the values for the



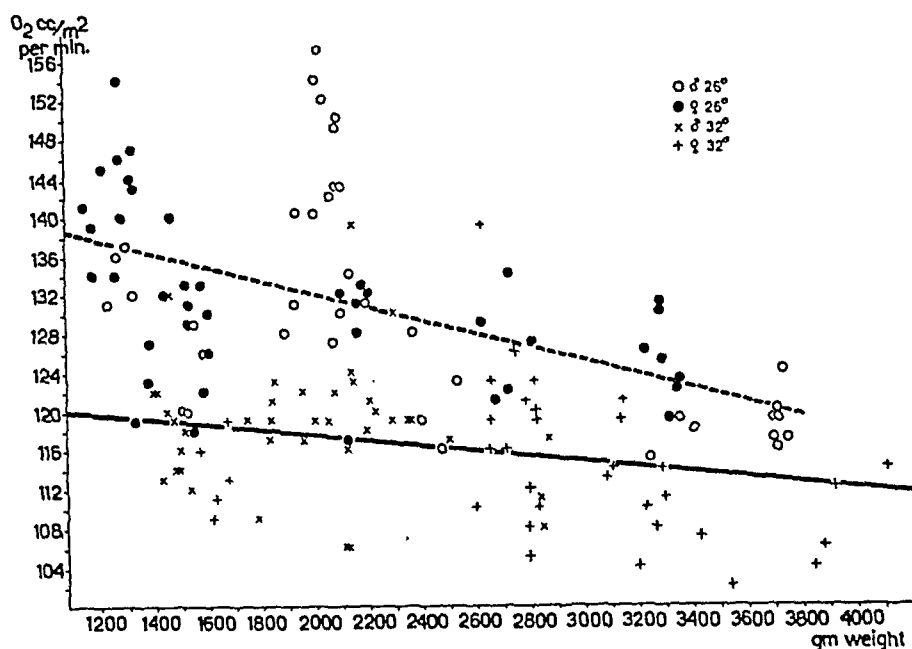


Fig. 3. Effect of weight, temperature and sex on oxygen consumption of guinea-pigs. Abscissa: total weight of the group (4 animals). Ordinate: oxygen consumption in cc per m<sup>2</sup> per minute. Body surface computed according to the experimentally found formula for males and females. The regression line — — — —, the equation of which is  $Y = -0.00699X + 145.9$ , shows the connection between weight and oxygen consumption per m<sup>2</sup> at a temperature of 26° C, after elimination of possible sexual differences. The regression line — — — —, the equation of which is  $Y = -0.00274X + 122.9$  shows the corresponding relation at 32° C.

oxygen consumption per sq. metre of the body surface that it was affected by the temperature as well as by the sex and weight. In order, so far as possible, to isolate the effect of each separate factor, the material was subjected to statistical analysis according to the methods of covariance analysis (BONNIER and TEDIN *op. cit.* p. 156).

The procedure was briefly as follows:

The oxygen consumption per sq. metre was correlated with the weight of the groups. Through the "class mean" — consisting of the values for one of the sexes at a certain temperature —, lines of regression A, B, C, D were drawn, with a coefficient of regression representing the average for the 4 classes. Through the mean for the whole material a line E with the same coefficient of regression was also drawn. If the sex or temperature factor had had no effect on the oxygen consumption per sq. metre, the mean square deviation from E of the means for sex or temperature would not be significantly greater than the average deviation of the

Table 4.

*Statistical analysis of effect of temperature and sex on the oxygen consumption per m<sup>2</sup> according to different formulae for computation of body surface. S = surface, W = bodyweight.*

Variation relating to	Degrees of freedom	Mean square	
		$S = 8.0 \cdot W^{\frac{2}{3}}$	$S_{\delta} = 8.22 \cdot W^{0.655}$ $S_{\text{♀}} = 9.86 \cdot W^{0.620}$
Deviation of the temperature means from the line of regression E .....	1	5973	6367
Deviation of the sex means from the line of regression E .....	1	745	3.3
Deviation of the group means from the lines of regression A, B, C and D .....	20	179	202
Quotients	Degrees of freedom	Variance ratios	P.
A. $S = 8 W^{\frac{2}{3}}$			
temp./groups .....	1/20	33.4	< 0.001
sex/groups .....	1/20	4.2	0.05
B. $S_{\delta} = 8.22 \cdot W^{0.655}$ $S_{\text{♀}} = 9.86 \cdot W^{0.620}$			
temp./groups .....	1/20	31.5	< 0.001
groups/sex .....	20/1	61.2	0.8—0.9

mean value for each group in each class from the lines of regression (A, B, C and D) of these classes. The results of the analysis can be surveyed in Table 4, which indicates that there was a significant difference ( $P < 0.001$ ) between the mean values for temperature and a probable difference ( $P = 0.05$ ) between the sexes in this respect, as the males had a 4 % higher oxygen consumption than the females.

A further analysis of the regression coefficients for the different temperatures and for the two sexes may also be made with covariance analysis. For the sake of perspicuity, however, this analysis has been made with the t-test, the results of which can be surveyed in Table 5. As the table shows with statistical significance, the oxygen consumption per sq. metre both at 26° and at 32° C was negatively correlated with the body-weight, that is to say,

Table 5.

The coefficients of regression with their standard error and the coefficients of correlation for the connection between the oxygen consumption per  $m^2$  of body surface and the weight at different temperatures, according to sex and different methods of estimating the body surface. The regression coefficients indicate in *cc* the decrease of the oxygen consumption per  $m^2$  when the total body weight of the groups increases by 1000 grams, i. e. when the weight of one guinea-pig increases by 250 g.  $P_1$  indicates the probability that the correlation was due to chance,  $P_1$  that the difference between the regression coefficients was likewise accidental.  $S$  = body surface,  $W$  = body weight.

Variate	Degrees of freedom	Body surface according to: $S = 8.0 \cdot W^{\frac{2}{3}}$			Body surface according to: $S \delta = 8.22 \cdot W^{0.655}$ $S \varphi = 9.86 \cdot W^{0.620}$		
		Coefficients of regression	Coefficients of correlation	P	Coefficients of regression	Coefficients of correlation	P
Males 26° C .....	36	-8.4 ± 1.97	-0.5808	< 0.001	-8.0 ± 2.08	-0.5388	< 0.001
Females 26° C .....	40	-8.2 ± 1.40	-0.6532	< 0.001	-5.9 ± 1.56	-0.5160	< 0.001
Males 32° C .....	37	-2.4 ± 2.42	-0.1609	0.3-0.4	-1.8 ± 2.57	-0.1118	0.4-0.5
Females 32° C .....	33	-4.8 ± 1.71	-0.4373	0.001-0.01	-3.2 ± 1.90	-0.2787	0.1
Within 26° C after elimination of sex differences = $b_{2s}$ .....	78	-8.3 ± 1.24	-0.6120	< 0.001	-7.0 ± 1.28	-0.5249	< 0.001
Within 32° C after elimination of sex differences = $b_{3s}$ .....	72	-4.1 ± 1.38	-0.3295	0.001-0.01	-2.7 ± 1.48	-0.2128	0.05-0.1
In males after elimination of temp. differences = $b_{\delta}^1$ .....	75	-7.2 ± 1.47	-0.4927	< 0.001	-6.7 ± 1.53	-0.4472	< 0.001
In females after elimination of temp. differences = $b_{\varphi}^1$ .....	75	-6.9 ± 1.13	-0.5771	< 0.001	-4.9 ± 1.20	-0.4271	< 0.001
Difference between:				$P_1$			$P_1$
$b_{2s} - b_{3s}$ .....	149	4.2 ± 1.85	—	0.01-0.05	4.3 ± 1.95	—	0.01-0.05
$b_{\delta}^1 - b_{\varphi}^1$ .....	149	0.3 ± 1.85	—	0.8-0.9	1.8 ± 1.98	—	0.3-0.4

the oxygen consumption per sq. metre diminished according as the body-weight increased. The regression coefficient, however, was found, with statistical probability, to be smaller at the higher temperature. It was thus evident that the oxygen consumption per sq. metre of the body surface was not constant in guinea-pigs of different weight and sex if the area of the body surface was computed according to Vierordt's formula. The explanation was either that the law regarding the constancy of the metabolism relatively to the area of the body surface did not apply strictly to guinea-pigs, or that Vierordt's formula did not correctly show the relation between the weight and the body surface in guinea-pigs.

Table 6.

*Weight and area of body surface in 16 guinea pigs.*

Females		Males	
Weight gm	Surface cm <sup>2</sup>	Weight gm	Surface cm <sup>2</sup>
216	284	178	246
315	332	350	384
324	359	437	432
482	454	542	489
642	566	654	600
758	575	778	696
955	664	848	665
983	751	878	660

In order to find out which of these two alternatives was right, 16 guinea-pigs, 8 of each sex and varying in weight from 178 to 983 grams were examined with special regard to the area of the body surface. The figures will be found in Table 6.

For the continued analysis, the logarithms for the body surface and the weight were computed and correlated. The following connection between the two factors was then found: for males,  $S_{\text{♂}} = 8.22 \cdot W^{0.655}$ , for females,  $S_{\text{♀}} = 9.86 \cdot W^{0.620}$  and for the whole material,  $S = 9.03 \cdot W^{0.637}$ . With shellack-fixed skins KIBLER, BRODY and WORSTELL (1947) found the formula  $S = 9.04 \cdot W^{0.64}$ . The correspondence is surprisingly good.

According to these experimentally found formulae, females as from a weight of 250 grams have a smaller extent of body surface than males of the same weight. At a weight of 1,000 grams the body surface of the males is 6 % larger in extent than that of the females. As compared with Vierordt's formula, it has been found that small animals have, relatively speaking, a somewhat

Table 7.

*Variation within groups of 4 guinea pigs as a function (1) of the length of time for which the oxygen consumption was recorded and (2) the interval between the observations. If these values are doubled, we get an approximate idea of the variation in individual animals.*

Source of variation	Degrees of freedom	Coefficient of variation
Between periods of 5 min. within half an hour ..	250	5.7
Between periods of 60 min. within 4 hours.....	24	2.6
Between periods of 60 min. within 3 days .....	32	4.1
Between periods of 60 min. between 14 days...	13	5.1
In female groups within 30 days .....	70	3.9
In male groups within 30 days .....	54	4.7

larger body surface than large animals. If the body surface is computed according to the formula:  $S = 9.03 \cdot W^{0.637}$ . The deviation from Vierordt's formula ( $K = 8.0$ ) is at a weight of 250 grams — 4 %, at 500 grams — 6 % and at 1,000 grams — 8 %.

As these results indicated that the deviations from the law regarding the constancy of the metabolism per sq. metre of the body surface were in part merely apparent, the oxygen consumption per sq. metre of the body surface was re-reckoned in accordance with the above experimentally derived formulae for males and females. The material was then analyzed in the same way as before (Tables 4 and 5).

It was found in this recalculation that the difference between the various temperatures was still statistically significant. On the other hand, no difference was found between the two sexes in regard to the oxygen consumption per sq. metre of the body surface. The regression coefficients likewise showed some diminution. Whereas the correlation between the oxygen consumption per sq. metre and the weight was found to hold good at 26° C, this was no longer the case at 32°. From Table 5 we also see that the coefficient of regression is less at 32° C than at 26°. The implication is that, if the surrounding temperature is lowered from 32° C to 26°, the oxygen consumption of a guinea-pig weighing 250 grams will be increased by 15 %, at 500 grams by 12 % and at 1,000 grams by 9 %.

The variation in the groups of four has also been computed for observation periods of different length, the results being shown in

Table 7. As we see, the coefficient of variation is least between 60 minute periods within four hours. This signifies that it is advantageous to determine the basal metabolism, if possible, in the course of the same day as that on which the effect of a factor on the metabolism is investigated.

Another question that called for investigation was whether the variation within the groups was modified according to weight, temperature or sex. It was found that only the sex possibly had any effect in this respect, in that the females showed an approximately 1 % lower coefficient of variation than the males. The difference, however, was not statistically significant.

It might be supposed that the individual variation was largely due to different motor activity. This, however, does not seem to be the case. The coefficient of variation for the oxygen consumption, measured in periods of 5 minutes, was for a series of non-narcotized guinea pigs  $5 \% \pm 0.55$  whereas when the animals had been narcotized with amytal the coefficient of variation was  $4 \% \pm 0.45$ . The number of 5 minutes periods in both cases was 50. There is no significant difference between these values. It may therefore be presumed that, at any rate for guinea pigs, the variation in the metabolism is due merely in a minor degree to differences in motor activity.

For the whole material, the coefficient of variation between different groups was 11.4 %, which is indicative of marked individual variations.

### Discussion.

As shown by the above remarks, considerable deviations from the law regarding the constancy of the basal metabolism per unit of body surface may apparently arise partly because the determinations are not made at thermoneutrality, partly because Vierordt's formula does not correctly show the real area of the body surface. Thus, a guinea pig weighing 250 grams has on an average an approximately 25 % higher oxygen consumption per sq. metre of the body surface than one weighing 1,000 grams, if the determinations are made at 26° C and if the area of the surface is computed according to Vierordt's formula. If, however, the area of the surface is estimated in accordance with the experimentally found formulae and if the tests are made at 32° C, it will be found that the difference is merely 8 % and is no longer statistically significant.

Similar conditions presumably apply also to animals other than guinea pigs. According to BENEDICT and MACLEOD (1929), the metabolic rate per sq. metre increases in growing rats with advancing age and increasing weight. The extent of the surface was computed by those authors according to Vierordt's formula. On the basis of figures given by GELINEO (1933) for the extent of the surface of growing rats, the present author has estimated the formula for the ratio of the body surface to the weight:  $S = 7.96 \cdot W^{0.74}$ . According to this formula the larger animals have a relatively larger surface than is shown by Vierordt's formula. The greater metabolic rate per sq. metre in the heavier animals seems therefore, at any rate in part, to be merely apparent.

Even in those cases where there seems to be a difference between males and females in regard to the metabolic rate per sq. metre, it would be safer to test the correctness of Vierordt's "weight and surface formula". The value of K in that formula is discussed by BENEDICT and FOX (1933) in a paper concerning determination of the body surface in mice. The mean value is estimated by them to be 8.78 for females and 9.15 for males. The possibility that the difference may have been due to chance has been estimated by the present author to 0.01—0.05, which indicates that it is statistically probable.

The fact that in small guinea-pigs the metabolic rate per sq. metre on a fall of the external temperature is more rapid than that of larger animals seems to be connected with their thinner fur covering and smaller subcutaneous layer of fat, owing to which they are not so well protected from heat loss.

No difference between the sexes with respect to the metabolic rate per sq. metre has been found in the present investigation. KIBLER, BRODY and WORSTELL (*op. cit.*) indeed state that the metabolic rate is about 4 % higher in female guinea-pigs than in the males. Those authors, however, compare merely four animals of each sex and the difference found does not seem to be statistically verifiable.

Though no statistically significant decrease in the rate of the basal oxygen consumption per sq. metre on increase of the body-weight at thermoneutrality could be detected by the present author as regards guinea-pigs, it may nevertheless be presumed that it occurs, as is borne out by the work referred to in the immediately preceding paragraph. Those three authors consider it desirable to express the relation between the total metabolism

per 24 hours and the body-weight by the ratio: metabolism per 24 hours =  $K \cdot \text{weight}^n$ , where  $K$  and  $n$  are determined from the correlation between the logarithms for the metabolism and the weight. The regression, however, will not be linear: it should be noted, instead, that between a weight of 50—500 grams,  $n$  is 0.67—71, but over 500 grams 0.39—0.63. The implication is that the regression between the oxygen consumption per sq. metre and the body-weight is probably not linear either. Endeavours have therefore been made to ascertain by a certain procedure (YULE and KENDALL 1947, p. 455) whether the regression at 32° C between the oxygen consumption per sq. metre and the weight may be satisfactorily represented by a straight line. The probability that the deviations from a linear regression were due to chance was found to be 0.1—0.2, whence there are no valid reasons for questioning the linear regression.

But, though the metabolism per sq. metre of the body surface probably shows some diminution with increasing weight, this diminution, relatively to the marked individual variations, is so slight that the law regarding the constancy of the metabolic rate per unit of body surface may nevertheless well correspond to the conditions in guinea pigs. This law has at any rate a biological background and may therefore be applied in preference to computation of the purely mathematical relations between the metabolic rate and the body-weight.

### Summary.

The variations found in determining the basal metabolisms are so considerable both as between different individuals and in the same individual on different occasions that it often involves considerable labour to show statistically the effects of drugs or other factors on the metabolism.

In order to diminish the labour involved in such attempts, the author indicates a procedure in which, after setting out from determination of the total metabolism of several animals taken together, a mean value for the individual animals is found by dividing the total metabolism by the number of animals. This will apparently reduce the variation. The advantages and drawbacks of this procedure are discussed.

The effects of temperature, sex and weight on the oxygen consumption of guinea-pigs were investigated in the way thus indi-



cated. The oxygen consumption per sq. metre, as we found, increased by 9—15 % when the temperature fell from 32° C to 26° C, it being noted that the oxygen consumption of small animals increased relatively more than that of larger animals.

If the body surface was estimated according to Vierordt's formula: body surface =  $K \cdot \text{weight}^{\frac{2}{3}}$ , the result indicated that males had a higher oxygen consumption per sq. metre than females; that at 26° C as well as at 32° the oxygen consumption per sq. metre diminished with increasing weight.

If, however, the body surface was computed by experimentally found formulae, no difference between the relative oxygen consumption of males and females could be noted. Nor at 32° were there any statistically significant differences between the oxygen consumption/m<sup>2</sup> of animals at different weights. At thermo-neutrality, therefore, the oxygen consumption/m<sup>2</sup> appears to be practically constant in guinea-pigs weighing between 250 and 1,110 grams.

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## The Effect of Two Wave-Lengths of Light upon the Same Retinal Element.

By

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The problem raised in this work concerns the interaction of two lights of different wave-lengths which simultaneously stimulate a retinal element which has been investigated in the eye of the decerebrate cat by means of the micro-electrode technique. Only large, well isolated spikes have been used and the animal has been dark adapted for not less than two hours with the micro-electrode inserted. This is our standard technique (see *e. g.* GRANIT, 1947).

In a way then we are dealing with the effects of colour mixture upon a single element. But the aim of such work on animals must be stated in terms of wave-length and impulse activity: here the question can be put very precisely with the aid of the schematic figure 1. We know that an element in the cat's eye may be excited by receptors of different colour sensitivities (GRANIT 1945, 1948, GRANIT and TANSLEY 1948, GERNANDT 1947, 1949). If two receptors overlap in spectral sensitivity in the manner illustrated by fig. 1 and we are stimulating by a single wave-length in the region of the

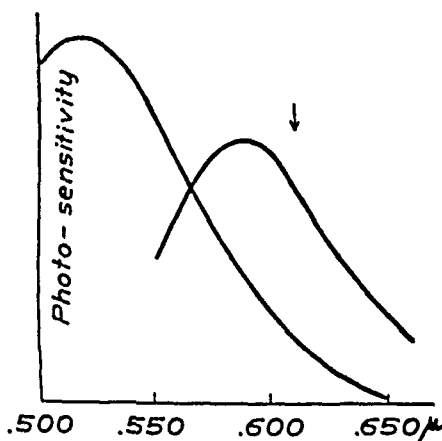


Fig. 1. Diagram showing the overlapping hypothetical absorption curves of two photosensitive substances in the retina (see text).

arrow in the figure, do we then always obtain merely a photochemical summation of the effects due to the addition of the two ordinates in the figure or is it, for instance, possible for either of the two responses so to 'canalize' the path that the other is wholly or partially suppressed?

The situation may be compared with the reflex effects of two different nerves upon the same motoneurone. When the impulses from these two sources enter the motoneurone, what happens? Is the output frequency of the motoneurone augmented or diminished? The photochemical overlap in the equivalent retinal experiment is a complication that always means addition of energy to both receptors but which can be minimized by selecting wave-lengths rather far apart.

The significance of possessing decisive information on this point is readily understood. Such information has been obtained in this work.

### The Technical Problem.

In order to give differentially sensitive receptors a good chance of interacting it is necessary to use supra-threshold stimuli which means that spike frequencies must be counted at onset and cessation of illumination. This being so it becomes imperative to know the result immediately both to be able to plan the experiment according to the properties of the element isolated and in order to find out whether the frequencies per unit time are stable enough for quantitative work. For these reasons automatic frequency counting was introduced and combined with an electronic shutter limiting the counting time to a duration of 1 or 2 seconds after respectively onset and cessation of a 3 or 5 sec. illumination. The total number of impulses during the counting period was directly and immediately read off during the experiment. Depending upon the stability of the preparation, from 3 to 8 illuminations were considered necessary to establish the count with an accuracy of within 10 %. If, owing to respiratory movements or other changes, the spike varied too much to render an accuracy of this order possible the result was rejected.

In the first version of this apparatus the calculator of a Geiger-Müller counter was connected to the condenser coupled amplifier from the micro-electrode over a relatively simple electronic shutter limiting the duration of the counting period. Later a clock-work counter, designed by Mr. B. AURELIUS of the Nobel Institute for Physics, was used. This ingenious mechanical device can easily follow up to 300 impulses/per sec. which proved satisfactory. An electronic delay and shutter, built by Mr. K. O. DONNER and E. ÅSTRÖM in this laboratory, was started by the amplified current of a photocell, placed in a de-

flected part of the stimulus beam of our Wright spectrometer (WRIGHT, 1946). Onset and cessation of illumination were led to separate channels of electronic delays and shutters adjustable so that the spikes at respectively 'on' and 'off' could be counted by two separate Aurelius clock-works delayed to any time after the action of the photocell, from about a few milliseconds to 5 seconds, and counting for durations which also could be selected within fairly wide limits. This convenient apparatus was necessary for some work of DONNER (1949) tracing time-frequency curves in detail. In my experiments it was set without use of the delay for instantaneous counting during 1 sec. after respectively onset and cessation of illumination, the eye in this case being exposed to the light only for 3 sec.

With all the wave-lengths and the full intensity range of the spectrum available the number of possible combinations of stimuli is legion. By means of the Wright colorimeter 2—3 wave-lengths can be applied singly or together onto the same retinal area in proportions selected by calibrated wedges. Clearly it becomes a most fundamental question how to select the particular stimuli and intensities to be tested, singly and together, in order to study the problem formulated in the introduction.

### The Theoretical Problem.

Since the spectral distribution of sensitivity of all elements, so far isolated in dark adapted retinae of cats (GRANIT, 1947) shows the influence of visual purple (VP) it is necessary to know the VP-curve of this retina with a high degree of accuracy. It has been shown that the pure on-elements are likely to be the purest VP-elements (GRANIT, 1947, GRANIT and TANSLEY, 1948, DONNER and GRANIT, 1949) whilst a considerable number of the on/off-elements, despite full dark-adaptation, have humps deviating from the smooth VP-distribution of sensitivity. The pure scotopic dominator which is the VP-curve of the on-elements, needed for this work, was carefully measured by DONNER and GRANIT (1949). This curve is slightly narrower than the VP-curve of extracts in solution. In comparing the effects of any two wave-lengths in the dark adapted eye we can measure the ordinates of energy of the two stimuli in the visual purple units obtained from the curve of DONNER and GRANIT (here called DG-ordinates) and thus know where we are in terms of the dominating photochemical substance in the dark adapted eye. The DG-ordinates thus represent reciprocals of the DG-curve, the latter being a plot of sensitivity (= reciprocal of energy) whilst for this work we have to return to the corresponding energy values in the spectrum.

If two wave-lengths are tested separately on a pure on-element and both are adjusted to equal DG-ordinates of energy, their responses, in terms of spike frequency, should be equal at all intensity levels if the receptors of the element concerned are pure visual purple receptors (also if other types of receptors are present but too few to influence the response or if actively inhibited). If this rule holds good, it also means that on/off-elements which behave in this fashion are dominated by visual purple to such an extent that they cannot be used to solve the problem we wish to solve.

*Experiment 1.* Inasmuch as one desires to use spike frequency as an index of colour effects it is imperative to know whether the statement just made is true or not. It has been repeatedly tested and found to hold good, in fact, I have never seen any deviations from this rule for pure on-elements. Now the range of spike frequencies in relation to stimulus intensity is, on an average, smaller for the on-elements than for the on/off-elements and this makes the test in experiment 1 less stringent than otherwise would be the case.

*Experiment 2.* This showed that the rule, tested in experiment 1, also held good for several on/off-elements. The test was generally carried out in either or both of two ways: (a) with a neutral filter interposed the frequencies were counted in a number of wave-lengths for equal DG-ordinates of energy. Then the filter was removed or further filters added. Again the rule was found to hold good both for 'on' or 'off' frequencies at all the wave-lengths selected. (b) two wave-lengths in the spectrum, generally  $0.520 \mu$  green and  $0.640 \mu$  red, were adjusted to equal DG-values, some multiple of unity for which the spike frequencies were identical on the counter. Then, adding the red and green and, simultaneously dividing the intensity for each of them by 2 so that the energy sum of the two remained equal to that of the individual stimuli, the frequencies should be constant. Several on/off-elements actually behaved in this way. The spike frequency for the sum of the halves was found equal to the spike frequency of the individual stimuli when our rule held good. This is a very sensitive test and therefore it became the standard method by means of which those on/off-elements were weeded out that to all appearance behaved according to the rule, i. e. as if they had been pure on-elements merely reflecting the properties of the homogeneous visual purple channel of reception.

Since full understanding of this argument is essential let us repeat it in slightly different terms: if only VP-receptors are involved the superposition of red and green (equated in energy for visual purple), each at half their former energy levels, will produce exactly the same photochemical effect as either full light alone. Therefore the response should be unchanged by this procedure. The on/off-elements that behaved in this way thus declared themselves to be unsuitable for the present investigation.

*Conclusion:* For pure on-elements and a considerable number of on/off-elements the curves relating impulse frequency to log stimulus intensity in the fully dark adapted eye must be independent of wave-length, provided that such curves are plotted in DG-units representing the visual purple distribution of sensitivity. This conclusion is simply a corollary of the experiments 1 and 2. This corollary, besides, was sometimes directly tested by plotting the appropriate curves from the data. But experiment 2 (b) gave the same result far more quickly and was just as reliable if a few more wave-lengths were added from the other end of the spectrum. It did not prove necessary to measure a large number of curves in different wave-lengths.

The DG-ordinates of energy are thus accurate enough to represent stimulation of an element by those receptors that contain visual purple. It is, of course, impossible to neglect this highly sensitive photochemical substance and we therefore have to proceed along the course laid out above which implied elaboration of methods by means of which the VP-contribution to the stimulus frequency could be assessed. The safest way is clearly to make the two stimuli equal in terms of DG-ordinates knowing, as we do, from the experiments just reported, that if the frequency responses to these wave-lengths then are equal they will remain equal at all levels of intensity available in the spectrum.

If the responses differ in spike frequency for two stimuli of equal DG-ordinates then something else has been stimulated besides visual purple and the experiment becomes a great deal more interesting. To these cases we shall now turn our attention. All of them refer to on/off-elements.

### Spike Frequency and Stimulus Intensity.

The spike frequency-log intensity curves of the on/off-element in fig. 2 are plotted on DG-energy units as abscissae for  $0.620 \mu$

(red) and  $0.520 \mu$  (green), on-components in full, off-components in broken lines and it is clear that it is impossible to find a red on/off-response identical with a green one for any point on the abscissa barring the extremes. By sliding a vertically placed ruler along the abscissa this will immediately become evident. Here then is a case in which, despite equal DG-energy values, one

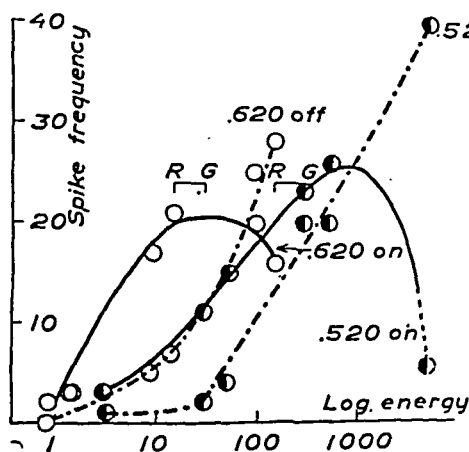


Fig. 2. Spike frequency-log intensity curves for on- and off-components of a red  $0.620 \mu$  and a green  $0.520 \mu$  stimulus. The logarithmic abscissae are in terms of multiples of energy necessary for visual purple threshold response.

The two places marked R and G in the middle of the figure show the energy levels at which the red and green were combined for simultaneous stimulation. The energies are obtained by projecting the vertical lines for R and G on to the abscissa.

wave-length can be distinguished from another by measuring the frequencies at 'on' and 'off' or their off/on-ratio. The element of fig. 2 belongs to a quite common type, the one for which the on-component is suppressed by inhibition in the high-intensity range while the frequency of the off-component still is rising. According to ADRIAN (1941) this kind of behaviour even dominates the high-intensity response of the whole optic nerve of the cat. It should be noted that in fig. 2 inhibition ultimately occurs in the on-component for both

wave-lengths and that, in general, the types of curve are similar though not superimposable by appropriate shifts along the abscissa. This, too, is characteristic of the cat's retina. Such curves can only be measured accurately by alternating between the wave-lengths selected so as to maintain the retina in the same state for the wave-lengths to be compared. Actually the curves illustrated are of a considerable degree of accuracy, 112 counts, representing 56 illuminations (counts doubled because of 'on' and 'off') averaged into 24 values. The high intensity inhibition for the green 'on' was observed on the screen and found to be almost complete but it could not be measured accurately because a high-threshold spike from another element with smaller spikes interfered in the counting machine. (Both loudspeaker



Table 1.

Energy ratio R/B	Red alone	Blue alone	Red + Blue
1400 .....	— 14	— 5	— 13
270 .....	— 8	— 6	— 10
180 .....	— 4	— 6	— 8
71 .....	— 3	— 6	— 9

and screen were always used to control the isolation of the spike as well as the response. Thus the spikes were both seen, heard and counted.)

The combinations R(ed) and G(reen), marked in the figure, were selected for simultaneous stimulation, the red being about one half the energy of the green. At the lower intensity-level red gave 21—7 for respectively 'on' and 'off', green 11—2. The sum of R + G gave 19—9. At the higher intensity level the red gave 16—28, the green 23—20. The sum R + G was 16—31. The interesting point here is that at the lower level of intensity, the absolute frequency of the red was higher at 'on' than that of the green and, vice versa, lower at the higher level of intensity. It is therefore clear that it was not the higher frequency as such that, as it were, took charge of the element when R and G were added but the *properties of the 'red'* that dominated over the *properties of the 'green'*. In both cases the "red reaction" determined the response of the element in the sum, both at 'on' and 'off'. It succeeded, so to speak, in dominating the path. The green was then made 150 times stronger than the red and now succeeded in impressing its value upon the sum.

It is difficult to evaluate inhibition quantitatively. It can only be done in cases where there is a high spontaneous frequency depressed by the light. This depression can be measured and the minus sign, used below, means the number of impulses subtracted per unit time from the spontaneous frequency owing to the inhibitory effect of the stimulus. Above is a case combining  $0.650 \mu$  (Red) and  $0.460 \mu$  (Blue) in which the latter was kept at a constant intensity and the former decreased in steps. Table 1 presents to the left the energy ratios Red/Blue. It is a case of inhibition of the on-component of an on/off-element. The spontaneous frequency was 16 per sec. and the decrease of this rhythm at the onset of illumination is tabulated.

The blue light is always kept the same, and the inhibition due

to it varies but little. As the red is weakened so the inhibition by it alone weakens but that of the sum  $R + B$  is practically constant in the last three horizontal rows. In these the inhibition for the sum exceeds that of the singles. Only the strongest red of the uppermost row was of the relative order of magnitude necessitated by the DG-scale. In all the other rows the red was far too weak relative to the blue so that red receptors must have contributed heavily to the element, in every case reinforcing the inhibition of the blue.

One generally tries to avoid the elements with heavy spontaneous frequency because the rhythm rarely keeps constant and for this reason I have comparatively few cases in which inhibition could be expressed quantitatively. I shall take another very striking case of inhibition of the on-component. Wave-length  $0.640 \mu$  (R) on the DG-ordinates gave an inhibition of  $-20$ . When pitted against the equivalent DG-values for  $0.420$ ,  $0.500$  and  $0.520 \mu$  the inhibition at "on" were respectively  $-16$ ,  $-14$  and  $-14$  whilst each of the three individual stimuli had given respectively  $-5$ ,  $+5$  and  $-3$ , all on the DG-ordinates. There was a progressive change in this experiment because  $0.520 \mu$  later gave  $+5$ . A number of other intermediate wave-lengths also gave values of that order. All these, when pitted against each other, gave values around zero or slightly positive values suggesting VP-dominance from  $0.600$  to  $0.420 \mu$ . But the extreme red in  $0.640 \mu$  always had this remarkably strong inhibitory component, clearly due to special receptors, that maintained their preponderance in any combination.

### Dominant Wave-Lengths in Mixtures.

*The theoretical problem.* I shall now proceed to another type of experiment in which all spectral stimuli always are at the same constant multiple of the visual purple curve in DG-units. For all wave-lengths the wedges are set for the DG-curve and the spectrum is used without additional filters. This means, about 200—500 times the threshold strength depending upon the sensitivity of the element. For the sum of red and green we use the half filter in the beam so as to keep the energy sum constant in terms of visual purple. It is clear that red and green enter into the energy sum, each with their half values, because of the half-filter interposed. If there is complete mixture the mean value might be obtained. If either of the two dominates in the response the value of the dominant wave-length at half intensity will show up unless the spectral curves of the substances represented in the two wave-lengths have a zone of overlap adding something to the

Table 2.

	G, full	G, half	R, full	R, half	Half R + G
On—off .....	27—16	21—9	25—30	21—16	23—18
Off/on-ratio .....	0.6	0.4	1.2	0.8	0.8

effect of the dominant wave-length. In any case it is clear that for a detailed analysis it is necessary to know the full values of the singles, their half values and the sum of the latter. With only visual purple present the sum of the half values will equal either full value and this is the situation already discussed above (Experiment 2) on p. 284.

*Experiment.* These schematic deductions are easily translated into an experiment which, accordingly, consists in determining the spike frequencies for  $R = 1$ ,  $R/2$ ,  $G = 1$ ,  $G/2$  and  $\frac{R + G}{2}$ . Above are tabulated the results of an actual experiment with  $R$  ( $0.640 \mu$ ) and  $G$  ( $0.520 \mu$ ), on an average 7.4 counts (= illuminations) for each figure. Counting period is 1 sec.

Within the limits of accuracy obtainable in such experiments it is clear that half the sum of the red and the green has reproduced the half-red value. The off/on-ratios show this very strikingly. They are identical and twice the value of the green off/on-ratio for the half value.

If the red path in this manner 'canalizes' the element when pitted against some other path it is often possible to stimulate the dominant path by some fraction of the energy required for the DG-ordinates and yet to obtain dominance in the sum. In another element the red ( $0.640 \mu$ ) full value for on-off, counted during 1 sec., was 44—37, its half value 39—32. The green ( $0.520 \mu$ ) full value 42—24, its half value 41—15 and the sum of the half values 44—33. There were on an average, 7.4 counts for each value and the accuracy was about 10 %. The on-component thus did not change much and behaved as if it had been dominated by visual purple but the red half value for the off-component came out in the sum. Then the experiment was repeated with the red stimulus reduced in strength to one quarter of its earlier DG-value. The result was: for the new red full value 44—29, its half value 45—20,  $G$  full value 43—25,  $G$  half value 41—15, and the sum of  $\frac{R + G}{2}$  gave 43—29. Into this sum red entered

with  $1/8$  of its original strength and yet it not only dominated the result for the off-component but this component (29) actually was greater than the amount (20) which the red half value could have contributed alone, not to mention the green half value that was only 15. In combination with  $1/2$  G the  $1/8$  R actually produced an off-effect corresponding to  $1/4$  R. This probably signifies that in this element the red modulator also to some extent was stimulated by the green stimulus. By reducing the strength of the red stimulus its frequency-intensity curve reached a portion of steeper rise (as can be seen by plotting the red off-values given above) so that it became a more sensitive index for red than at the higher intensity level.

*Conclusion.* By these experiments the principle has been established that in a combination of two wave-lengths one of the two may be prepotent and impress its particular distribution of on- and off-frequencies upon the sum. Such facts can, of course, only be established when the two wave-lengths differ in their individual effects so as to provide us with an index for measuring the dominant and the suppressed wave-length.

### Tests with Other Wave-Lengths.

We proceed to observations with elements in which various wave-lengths were used at their DG-values at the maximum intensity of the spectrum and the half filter employed for the sum so as to keep the energy sum constant in terms of visual purple. The first spike to be mentioned is one with post-excitatory inhibition in the green at  $0.520 \mu$  for which the on-off values were 8— — 1, *i. e.* a slight suppression of the spontaneous activity at 'off'. The  $0.640 \mu$  red gave 11— + 1 the sum  $\frac{R + G}{2}$  7— — 1, *i. e.* the value of the green. In the same experiment the violet  $0.420$  alone gave 8— + 1 and combined with the green 10— — 1 perhaps summation at 'on' but preponderance of the green post-excitatory inhibition at 'off'.

A very perfect experiment is illustrated by Table 3. There was practically no spontaneous rhythm. The green  $0.520$  varied between on-off-values 18—4 and 19—5 during a couple of hours while it was combined with lights in a number of other wave-lengths, all values adjusted to the DG-curve, the sums obtained

Table 3.

Wave-length in $\mu$	0.640	0.620	0.600	0.580	0.520	0.460	0.440	0.420
Singles on .....	25	24	24	19	18—19	26	16	23
off .....	11	10	7	5	4—5	5	4	7
Combined with 0.520 $\mu$ on.....	19	20	20	20		18	20	20
off .....	7	7	5	4		3	5	6

with the half filter. There were 5—6 counts for each value, 2 sec. counting period.

From 0.640 to 0.600  $\mu$  the off-effect diminishes towards the middle of the spectrum. The combination with 0.520 regularly brings out the value of the green on-component whilst the red off-component maintains itself somewhat better and approaches a mean value between the constant green and its own wave-length. In 0.600 and 0.580  $\mu$  the green 0.520 already dominates in the sum. In the short wave-lengths there is a sudden rise of the on-component in 0.460  $\mu$  but this is suppressed in the combination with the green. The same happens to the drop of the on-component in 0.440 and the new rise in 0.420. The green 0.520 maintains itself as the dominant wave-length in every match, and there is a measurable sign of something else at 'off' only in combinations with the extreme red and violet. Increased off-effects in the short wave-lengths are quite common. Sometimes they are maintained against a low off-value in the green (0.520  $\mu$ ) but more often than not the green is relatively more dominant in combinations with blue and violet than in combinations with red.

It is perhaps not necessary to present further examples. Suffice it to mention that in an experiment similar to the one of Table 3 the green (0.520  $\mu$ ) again was combined with the same short and long wave-lengths and that the value for the off-effects fell practically on the mid point between the low green off-effect (19) and the others which varied from 42 to 21 with maxima in 0.640 (42) and 0.440  $\mu$  (30). In this case there was no dominance but perfect mixture of the two effects. The on-component varied but little from wave-length to wave-length.

Red modulators are common and by several methods easy to demonstrate and were therefore well suited for the demonstration of the principle that has been established. I often tested the violet in 0.440 or 0.420  $\mu$  in the cases where experiments were performed with the combination red and green and noted that, if the off-

effect was stronger for red than for green (DG-units), then it nearly always had a small rise in the violet too. This observation is merely mentioned in passing and I do not intend to discuss alternative explanations of it.

### Discussion.

The results obtained are, of course, dependent upon the use of the DG-ordinates as units of energy. Despite this uniform scale there have been qualitative differences in interaction from case to case although red dominance has been easier to demonstrate than prepotency of other wave-lengths. Activation of red modulators is favoured by the increase of energy necessary to compensate for the low sensitivity of visual purple in the extreme red. Nevertheless green dominance has been seen from time to time (Table 3) and, also, if, in red dominance, the red stimulus is shifted in steps from  $0.640 \mu$  towards the green at  $0.520 \mu$  it is but rarely that in  $0.580 \mu$  the red component succeeds in showing up against the green component of the stimulus whatever the nature of the latter. To maintain prepotency the red modulator generally must be stimulated within  $0.640$  to  $0.600 \mu$ . The best effects have been obtained with the red at  $0.640 \mu$ .

Once more I want to draw attention to the interesting fact that, when the red end is characterized by an increased off-response, so also is the violet at  $0.420 \mu$  though in this region the increase generally is somewhat smaller. GERNANDT (1948) determined the off/on-ratios at the threshold of the dark adapted eye for 72 elements and in this material found the probability for maximum off/on-ratios to be only 0.18 in the green as against 0.47 in the red and 0.39 in the blue. The off/on-ratio in his work was calculated on threshold sensitivity, *i. e.* reciprocal of threshold energy. In a later set of experiments of the same type GERNANDT (1949) measured the off/on-ratios of a number of elements in the whole spectrum and found some for which the values increased towards the two ends.

The animal may or may not discriminate with the aid of the off/on-ratio as such. The significant point in our measurements is that the variations in relative spike frequency at 'on' and 'off' serve to indicate differences in the rates of rise and fall of the frequency-log intensity curves for different wave-lengths. When an

animal turns its eye towards the coloured light these differences will emerge in the pulsations of frequency set up by the stimulus.

The demonstration that two wave-lengths may interact in such a fashion that, to judge by the frequency pattern, one is pre-potent and the other one suppressed is of particular interest against the far less striking evidence for the opposite process, a facilitation leading to pooling of the effects of the two wave-lengths at 'on' and 'off'. Within a considerable range of relative intensities the elements are thus easily triggered in favour of either of two wave-lengths in a most interesting alternative fashion. The explanation may be that the wave-lengths used in this work have been rather far apart and that for this reason we have struck the effects which psychophysically are known as contrast phenomena. These are characterized by that same aspect of exclusion of one alternative in favour of another. We shall proceed to test this proposition by successive illuminations. The selection of wave-lengths in this work has been determined by the desire to create a situation that could be analyzed. Phenomena of summation would provide greater difficulties if, as seems probable, they would require wave-lengths that did not differ by specific signs.

These experiments, as far as they go, are not without interest for our ideas about colour mixture. The final mixing of colours may well be a central process but the retina itself has here been shown to deliver a message determined by synaptic interference with consequent suppression or enhancement of one part of the spectrum. There are therefore interesting retinal problems of interaction involved in colour mixture. For this reason negative coefficients in work on colour mixture as well as deviations from Abney's law seem perfectly reasonable.

### Summary.

Well isolated elements picked out by the micro-electrode technique from the dark adapted retina of the cat have been stimulated with one or two wave-lengths of a spectrum and the discharge of spikes counted at 'on' and 'off'.

In many isolated elements the log intensity—spike frequency curves have been shown to run a different course for different wave-lengths. When this is so two wave-lengths can be shown to elicit different relative frequencies at 'on' and 'off' within a large

range of variation in the energy ratio of the two wave-lengths. The pure on-elements and a considerable number of on/off-elements do not have these properties; wave-lengths equated for equal visual purple activation at one energy level then remain equated at all intensity levels.

When two wave-lengths, which *can* be differentiated by their relative spike frequencies at 'on' and 'off', are acting simultaneously on the same element it is generally found that either of the two is prepotent and succeeds in maintaining its off/on-ratio in the sum whilst the properties of the other one are suppressed.

By making such comparisons in equal retinal visual purple units the contribution of visual purple stimulation can be kept constant for all wave-lengths to be combined. By these means contributions from other types of receptors to the interaction patterns of two wave-lengths can be distinguished.

Several examples of interaction between the effects of two wave-lengths upon the same element are given. They substantiate the conclusion that interaction can take place when one wave-length simultaneously stimulates two photochemical substances with ordinates overlapping the same spectral region.

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## Anti-Sympathetic Action of Sympathomimetic Amines.

By

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Since BARGER and DALE (1910) published their classical investigation of what they called sympathomimetic amines much work has been carried out to determine the different actions of these compounds which with greater or lesser fidelity imitate the effects resulting from stimulation of the sympathetic nerves.

There are, however, some observations which indicate that some of these amines also possess properties of a different variety. For example, FINKLEMAN (1930) demonstrated that ephedrine was capable of abolishing the relaxing effect of adrenaline. He also showed that ephedrine suppressed the relaxation produced by electrical stimulation of the periarterial nerves to the rabbit intestine. The antagonistic action of ephedrine with respect to adrenaline has also been studied by BURN and TAINTER (1931), THIENES and coworkers (1934) and others.

This anti-sympathetic effect is not specific for ephedrine. Thus for instance some earlier experiments in this laboratory showed that 1-N-ethylephedrine had qualitatively the same action (ÅSTRÖM 1948).

The experiments to be described were carried out with the purpose of investigating whether this anti-sympathetic action was a common characteristic for all related amines belonging to the sympathomimetic group, and if so, what conclusions could be drawn as to the correlation between chemical structure and anti-sympathetic effect.

## Method

Isolated strips of rabbit ileum were used for the experiments. The strips were excised with the preservation of the periarterial nerves and suspended in Tyrode's solution. The nerves were placed upon a pair of stimulating electrodes. The bath was kept at 38° C and aerated with 6 per cent carbon dioxide in oxygen. For stimulation faradic current from an induction coil was used.

The sympathomimetic amines were tested in the following way. First the minimal effective dose upon the intestine was determined. The effect of these small doses for some amines was stimulation and for others inhibition. When thus the minimal effective dose was determined the dose was increased until the intestine was completely inhibited. For each experiment the doses used of the different amines were compared with the result of a corresponding dose of l-ephedrine and the relative figures were calculated by taking the dose of ephedrine for producing complete inhibition as 10.

When now the degree of activity of the amine in question was known its influence upon the sympathetic inhibition was studied. The nerves were stimulated and the well-known relaxation and inhibition of the rhythmical movements recorded. Before adding the amine the effect of adrenaline and l-nor-adrenaline was also checked. After that the minimal effective dose of the amine was added and the effect of electrical stimulation again tested. Usually no change could be observed. Hereafter the same dose of the amine was given repeatedly, each time followed by 10 seconds' stimulation of the nerves. This was continued until the effect of electrical stimulation was abolished. The dose of the amine used up to this point was related to a dose of ephedrine producing the same effect. The dose of l-ephedrine for producing complete inhibition of the intestine was taken as 10. When now the electrical stimulation of the nerves gave no response, adrenaline and l-nor-adrenaline were also without effect in doses which before adding the amine had a marked inhibitory action.

After washing the bath the nerves were again stimulated to make sure that a displacement of the electrodes or some other change in the experimental conditions was not responsible for the decreased effect of electrical stimulation. The usual relaxation could always be produced again, even though on some few occasions the bath had to be washed several times. When the stim-

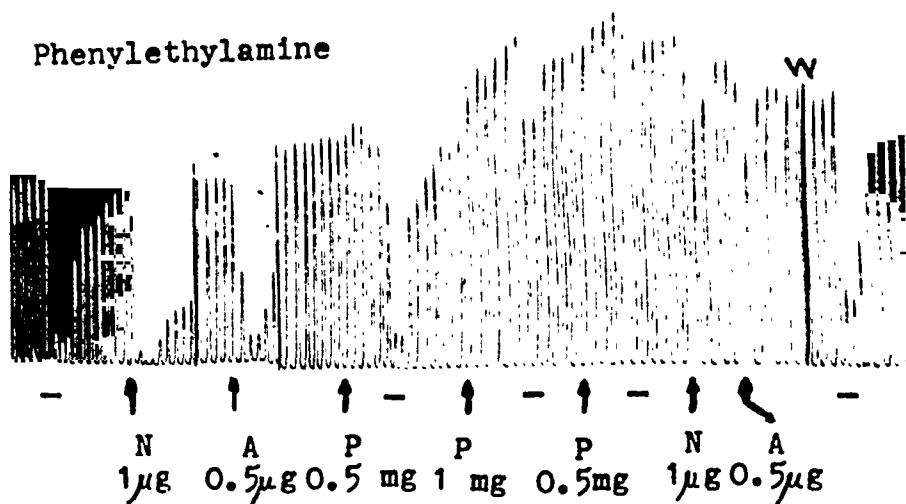


Fig. 1. Rabbit intestine. The figure shows the effect of adrenaline (A), l-noradrenaline (N), and electrical stimulation (—) before and after the adding of phenylethylamine (P) to the bath. Phenylethylamine antagonizes the sympathetic inhibition.

After washing (w) electrical stimulation has again the usual inhibitory effect.

ulation once more gave a normal response, adrenaline and l-noradrenaline had also regained their usual effects. — The principle for the determination is shown in figure 1.

The compounds not belonging to the group of sympathomimetic amines were tested along the same lines. Also here the sum of the repeated small doses were calculated in relation to l-ephedrine.

The amines were used in 1 per cent water solutions. Any adjustment of the pH was not made because it was found that no significant change in the response to electrical stimulation could be observed within the pH-range 6.8—7.8. The pH of the bath was 7.2 and the addition of the amine solutions never altered the pH more than 0.1 unit.

## Results.

### A. Effect of stimulation of the mesenteric nerves.

As mentioned above the effect of stimulation of the periarterial nerves to the intestine was a relaxation and a complete inhibition of the rhythmical movements. In a few instances, however, a stimulation was recorded. This contraction proved to be due to a direct spread of the electrical current to the intestinal muscle, because if the electrodes on those occasions were placed

more distant to the intestine this contraction could always be made to disappear.

Mc SWINEY and ROBSON (1931) investigated the effect of different types of electrical stimulation applied to the periarterial nerves of isolated strips of rabbit stomach and small intestine. They found that the response of the stomach depended upon frequency, strength, and duration of the stimulus. The isolated strips of the intestine, on the other hand, were relaxed by all types of electrical stimulation. In the experiments presented here these results proved to hold true for any kind of faradic current obtainable from the induction coil (frequency, 45—80 cycles per second; strength 10—60 v, as measured directly from the disconnected secondary.) The effect of various types of electrical stimuli applied to periarterial nerves to the isolated small intestines from different animals will be discussed more in detail in a subsequent article.

BROWN and Mc SWINEY (1932) showed that stimulation of the thoracic sympathetic trunk in cats gave an effect upon the stomach which varied with the type of anaesthesia. They studied particularly the action of luminal. It was found that under luminal anaesthesia relaxation of the stomach was almost never observed; an effect which under other forms of anaesthesia was very common. The ileum, however, was relaxed under all forms of anaesthesia. Because of these observations it now seemed interesting to study the effect of electrical stimulation of the nerves to the isolated strips of rabbit ileum in the presence of different barbiturates. In the experiments presented here different ethyl and phenyl barbiturates were investigated. No influence upon the effect of electrical stimulation could be observed even for large doses (approximatively 1:2000) where the barbiturates exerted an inhibitory action upon the rhythmical movements.

### B. *Sympathomimetic amines.*

The amines were classified according to their chemical structure as represented in table 1. It proved impossible to acquire representatives for all the main groups and only the fourteen amines mentioned could be examined.

The amines were investigated as to their antagonistic effect upon the relaxation produced (1) by adrenaline and l-nor-adrenaline, (2) by electrical stimulation of the periarterial nerves. The doses needed of the sympathomimetic amines to antagonize the two kinds of sympathetic relaxation were almost identical. No

Table 1.

DERIVATIVES OF	ALIPHATIC NUCLEUS	
	WITHOUT ALCOHOLIC -OH ON THE ALPHA CARBON ATOM	WITH ALCOHOLIC -OH
I.  Phenyl Amines	<sup>a/</sup> $\text{-CH}_2\text{CH}_2\text{NH}_2$ Phenylethylamine $\text{-CH}_2\text{CH}(\text{NH}_2)\text{CH}_3$ Benzedrine	<sup>b/</sup> $\text{-CH(OH)CH}_2\text{NH}_2$ Phenyl-oxyethylamine $\text{-CH(OH)CH}(\text{NH}_2)\text{CH}_3$ Propadrine $\text{-CH(OH)CH}(\text{NH-CH}_3)\text{CH}_3$ Ephedrine $\text{-CH(OH)CH}(\text{NH-CH}_3)\text{CH}_2\text{CH}_3$ N-ethylephedrine
II. Ortho-hydroxyphenyl Amines	Not available	
III. Meta-hydroxyphenyl Amines	<sup>a/</sup>	<sup>b/</sup> $\text{-CH(OH)CH}_2\text{NH(CH}_3)_2$ Neo-synephrin
IV. Para-hydroxyphenyl Amines	<sup>a/</sup> $\text{-CH}_2\text{CH}_2\text{NH}_2$ Tyramine $\text{-CH}_2\text{CH}(\text{NH-CH}_3)\text{CH}_3$ Paredrinol $\text{-CH}_2\text{CH}_2\text{N(CH}_3)_2$ Bordenine	<sup>b/</sup> $\text{-CH(OH)CH}_2\text{NH(CH}_3)_2$ Synephrin
V. Dihydroxyphenyl Amines	<sup>a/</sup>	<sup>b/</sup> $\text{-CH(OH)CH}_2\text{NH}_2$ Arterecol $\text{-CH(OH)CH}_2\text{NH(CH}_3)_2$ Adrenaline $\text{-CH(OH)CH}_2\text{NH-CH(CH}_3)_2$ Aludrin (Boehringer)

The amines were prepared and kindly put at my disposal by Astra Ltd, Södertälje, Sweden, and by Hoffman La Roche, Basel, Switzerland.

special emphasis was given to the slight differences that might have ensued. As described earlier, the technique used gave only an answer to the question whether adrenaline and nor-adrenaline were without effect when the relaxation produced by electrical stimulation was abolished. The principle for the determination is shown in figure 1, where the action of phenylethylamine is chosen as an example. The results obtained are presented in table 2.

The effect of the amines in some instances depended upon the method by which they were added to the bath. Thus, if one large single dose was given the intestine was often completely inhibited, while if the same dose was divided into fractions and added at intervals the total response was much less. This was regarded as an effect due to tachyphylaxis which is a well-known character-

Table 2.

Compound	Stimulation	Relative dose for Total inhibition	Anti- Sympathetic action
I.			
a) Phenylethylamine .....	2	20	8
Benzedrine .....	2	6	4
b) Phenyl-oxyethylamine .....	2	15	10
Propadrine .....	1	12	6
Ephedrine .....	2	10	7
N-ethylephedrine .....	1	4	2
III.			
a) —	—	—	—
b) Neo-synephrin .....	—	8	5
IV.			
a) Tyramine .....	1	30	20
Paredrinol .....	2	10	6
Hordenine .....	1	15	7
b) Synephrin .....	—	8	5
V.			
a) —	—	—	—
b) Arterenol .....	—	0.006	—
Adrenaline .....	—	0.003	—
Aludrin .....	—	4	—

The figures in the table are related to ephedrine the inhibitory dose of which is taken as 10. For further explanation see text.

istic for some of the compounds in this group of amines, *e. g.* ephedrine. If small doses of the amine were given during a longer period the decrease of action in some cases could also be due to oxidization. This was particularly true for group V b (table 1) where the amines were sometimes oxidized to adrenochrome-like substances, which gave the bath a reddish colour. The figures in table 2 represent an average value from the results where no sign of oxidization could be observed.

The amines could be divided into three main classes according to their action upon the rhythmical movements of the intestine. The first class (adrenaline, arterenol, and aludrin) caused only inhibition. The second class (phenylethylamine, benzedrine, ephedrine, ethyl-ephedrine etc.) had a slight and definite stimulating effect in small doses but inhibited the intestine in larger ones. The third class (tyramine and to some extent hordenine)

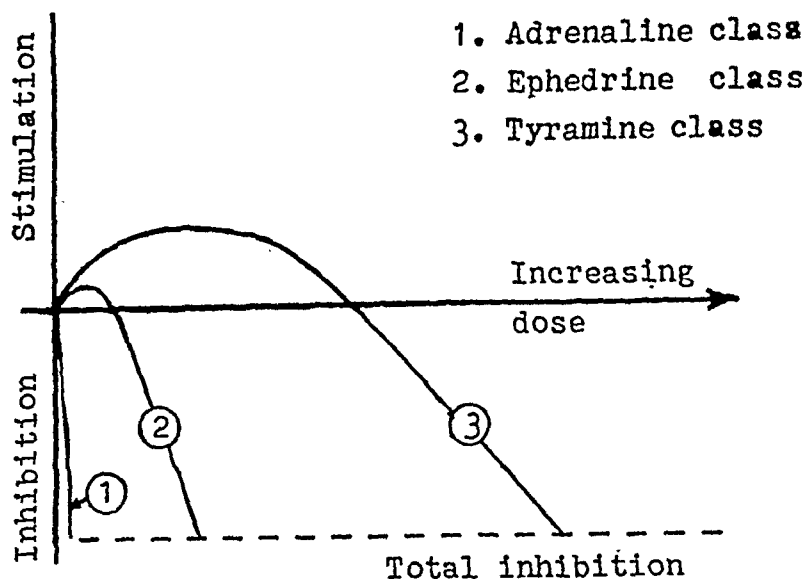


Fig. 2. Schematic representation of the action of the amines belonging to the three different classes discussed in the text.

had a marked stimulating effect and only in very large doses did they inhibit the intestine. This is illustrated schematically in figure 2. The relative doses for complete inhibition of the rhythmical movements of the intestine are given in table 2.

Table 2 shows that the sympathomimetic amines rather commonly antagonize the action of adrenaline, nor-adrenaline, and sympathetic stimulation. Any definite idea about the structure of a compound which should have a maximal antagonizing effect is difficult to ascertain on the basis of table 2. From the results it is clear, however, that hydroxyl groups in the benzene ring or on the alpha carbon atom of the aliphatic chain does not seem necessary for the antagonizing action.

In an effort to estimate the degree of specificity of the antagonism some other substances were examined. Barbiturates had no effect as has already been described. Atropine in doses sufficient to abolish the effect of 1  $\mu$ g of acetylcholine in the bath of 20 cc had no effect upon the sympathetic relaxation. Cocaine had no effect which is in agreement with BURN and TAINTER (1931). Morphine, Prostigmine (Roche), Substance P (EULER and GADDUM, 1931) and arecoline did not alter the sympathetic response, and the same was the case with acetylcholine and histamine.

As a representative for compounds with pharmacological actions similar to those of the sympathomimetic amines but of a

different chemical structure Privin<sup>1</sup> (Ciba) was examined. Also here an anti-sympathetic effect could be observed. The dose needed for complete antagonization of the relaxation brought about by electrical stimulation was 1—2 in relative figures (calculated as mentioned above).

Some known anti-sympathetic substances were also studied. The anti-sympathetic effect was easily demonstrable for ergotamine (Gynergen, Roche) and 933 F (Fourneau, piperidino-methyl-benzodioxane). The anti-sympathetic doses for these compounds were in relative figures approximatively 0.3—0.4.

### Discussion.

It has been shown in these experiments that the sympathomimetic amines rather commonly antagonize the relaxation produced not only by adrenaline and nor-adrenaline but also by electrical stimulation of the mesenteric nerves. For comparison it therefore seems interesting to consider the chemical processes involved in the sympathetic nervous transmission. The true nature of these processes is not fully understood. The rather complicated conception of an intermediate substance etc. propounded by ROSENBLUETH (1932) and by CANNON and ROSENBLUETH (1933) seems difficult to maintain in the light of recent discoveries. For the same reason the idea that the ergone is actually formed at the nerve endings must be doubted. In 1946 EULER demonstrated that the sympathetic nerves contain great amounts of a pressor substance chemically different from adrenaline, and has later brought forward evidence for its identity with l-nor-adrenaline (1948).

These results by EULER make it more likely that on stimulation of sympathetic nerves the "sympathin" already present in the nerves is set free at the nerve endings and thus reaches the effector cells (cf. EULER 1946). This concept is supported by some results previously obtained in this laboratory (EULER and ÅSTRÖM, 1948). It was shown that when the isolated splenic nerves from cattle were stimulated electrically, a substance acting like "sympathin N" (nor-adrenaline) was released at the cut end of the nerve. The observations by LISSÁK (1939) and others may be interpreted in the same way.

This concept of the transmission mechanism at the sympathetic nerve endings is partially in agreement with the idea of AHLQUIST

<sup>1</sup> 1-naphthyl-methylimidazoline hydrochloride.



The first "adrenaline class" included the amines in group V b, table 2. These amines caused only inhibition and it proved impossible to demonstrate any antagonistic action with regard to the relaxation produced by electrical stimulation of the periarterial nerves.

The second class, "the ephedrine class" according to figure 2, had less adrenaline-like action. Small doses of these amines stimulated the intestine, larger ones caused inhibition. In this case it was easy to demonstrate the antagonistic action with respect to the sympathetic inhibition.

The third class mainly represented by tyramine had still less adrenaline-like actions. The amine had a very strong stimulating effect and inhibition was observed only after very large doses. Even for the demonstration of the anti-sympathetic action large doses were needed.

From these observations it seems tempting to advance some general principle for the kind of pharmacological competition studied in these experiments. It seems reasonable to assume that if a compound should possess the capability to antagonize a certain compound it should be chemically similar enough to react with the same structures of a cell but on the other hand it must be different enough not to exert its action to the same degree. This could be regarded as a condition since otherwise it should be impossible to add the "competitor substance" in a sufficient concentration.

These aspects might also be applicable to the phenomenon of tachyphylaxis which is a well-known characteristic for the compounds in the ephedrine class. The observation that on repeated injections the effect of these amines becomes less and less might indicate that the first added quantities exert a competitor action with regard to the subsequent doses. It is not unlikely that the first added quantities in these cases have become chemically altered and thus fulfil the requirements for a "competitor substance" as discussed above.

It was stated above that the amines in the "adrenaline class" had no ability to antagonize the relaxation produced by electrical stimulation of the periarterial nerves. This statement is in opposition with the results by FINKLEMAN (1930). Using the same technique as here described he added small doses of adrenaline repeatedly at intervals until the intestine had come into a state of "equilibrium with a fairly high concentration of adrenaline".

When at this point the effect of electrical stimulation of the periarterial nerves was checked he did not get the usual relaxation. His results have been confirmed in our experiments. However, some observations indicated that the anti-sympathetic action recorded was not an effect of adrenaline per se. Thus for instance it was easier to demonstrate the anti-sympathetic effect of adrenaline if old solutions were used instead of fresh ones. Further it was observed that when the electrical stimulation had a lesser inhibitory action the bath often showed a slightly reddish colour, indicating that adrenochrome had been formed. Similar were the results with aludrin (group V b, table 1). An anti-sympathetic action could also here be demonstrated in some cases but this never occurred until the bath had a marked reddish colour indicating the formation of isopropyl-adrenochrome.

In the case of adrenaline it was tempting to assume that the anti-sympathetic effect was due to adrenochrome. To investigate whether this was the case some adrenochrome solutions were prepared by oxidizing adrenaline with iodine at pH 6. With this adrenochrome solution an anti-sympathetic action could sometimes be demonstrated. The dose needed, however, was much greater than in the case of adrenaline. This made it probable that the substance in the bath causing the anti-sympathetic action was not identical with adrenochrome but a substance representing an intermediate product in the oxidization process of adrenaline. Previously oxidized aludrin was also less effective than aludrin oxidized in the bath during the experiments.

It must be emphasized, however, that the experiment according to FINKLEMAN is difficult to perform and a "state of equilibrium" is not always possible to produce. For definite proof, therefore, more experiments are needed.

It was stated above that the sympathomimetic amines act directly upon the cell in the same manner as does adrenaline. This is not generally agreed to, however. Thus for ephedrine it has been stated by BLASCHKO (1938) that this compound, as well as other derivatives of phenylisopropylamine, acts as an inhibitor of the amine oxidase. GADDUM and KWIATKOWSKI (1938) have further advanced this theory and have compared the action of ephedrine to that of eserine. Their theory has not been generally accepted, however, and the existence of a specific amine oxidase is still subject to debate.

The experiments presented here lend no support to the concept that the mechanism of action should be different for the phenyl-isopropylamines (*e. g.* ephedrine) than for the other investigated amines. In all cases the effects studied are most coherently explained if the amines are supposed to act directly upon the effector cells as has earlier been discussed. Another observation which speaks against the amine oxidase theory was that d-,l-, and d- $\psi$ -forms of ephedrine were all about equally effective in the respects investigated here upon the rabbit intestine. The results with d- and l-forms of benzedrine were identical. If the amines should have exerted their action upon an amine oxidase some difference in activity should most likely have been present.

### Summary.

The effect of fourteen sympathomimetic amines has been studied on isolated strips of rabbit's intestine with special regard to their ability to antagonize the inhibition produced by adrenaline and l-nor-adrenaline as well as by electrical stimulation of the mesenteric nerves.

For the sake of discussion the amines have been divided into three classes and each class described.

The anti-sympathetic property earlier reported for ephedrine and l-N-ethylephedrine has proven to be a rather common characteristic of the sympathomimetic amines. The only amines which did not have the anti-sympathetic action were the dihydroxy-phenyl substitutes *i. e.* adrenaline, arterenol, and aludrin (BOEHRINGER). The anti-sympathetic action that was sometimes observed for adrenaline and aludrin has been ascribed to some derivatives formed during the oxidization of these compounds.

d- and l-forms of ephedrine and benzedrine have proven to be almost identical in their effects upon the intestine *per se*, as well as in respect to the anti-sympathetic action. This has been considered as an argument against the amine oxidase theory.

The anti-sympathetic action of the sympathomimetic amines has been briefly discussed upon a chemical basis. The idea is propounded that the anti-sympathetic action of these amines is an example of pharmacological competition.

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## **Regeneration of the Motor and Sensory Fibres in the Sciatic Nerve and the Suralis Nerve of the Cat.**

By

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In spite of extensive experimental research in animals the knowledge of the local powers directing regeneration is very elementary as regards the nervous system. Modern researchers have so far failed in discovering definite morphological signs of a guiding principle in the regeneration of the extremity nerves.

### **Factors Aiding the Establishment of Appropriate Peripheral Nervous Connections.**

It is difficult to find any signs of an orientation mechanism after a complete severance or neurotmesis of a mixed peripheral nerve in man or in animal under the usual, very intricate conditions. The interweaving of fibres at the suture line has been described. How growing nerve fibres enter suitable channels seems to be a matter of chance. SEDDON (1944) — a keen student of nerve regeneration — denied any selectivity controlling the formation of peripheral connections. He stated as follows: "Axon buds grow into any convenient Schwann-cell tubes, and there is no distinction between the behaviour of motor and sensory sprouts, a motor axon may grow into a Schwann tube connected with a sense organ and vice versa." YOUNG (1942) in a review emphasized the fact that the only probable mechanism for making proper connections between the two stumps is the provision of a

large number of new fibres. This phenomenon favours the accidental entrance of some sprouts into suitable channels. SANDERS and YOUNG (1942) also denied the attractive effect of degenerated nerve tissue, because they did not observe any marked difference when degenerated nerve was used to connect the ends instead of fresh tissue. According to WEISS (1934) the only remaining possibility is that a particular tissue may possess the power to select its corresponding nerve during embryogenesis but certainly not later. He has also shown that sensory fibres can make even functional connections with motor end-plates.

There have been many attempts to explain a certain regularity in the nerve regeneration observed by some investigators. The matter has been studied by means of resection technique — often in connection with tubing or transplantation — as well as by in-vitro experiments. The mechanical point of view is the earliest. VANLAIR (1882, 1885) found that growing fibres are directed towards the lowest mechanical resistance. This observation was disproved among others by WEISS who demonstrated the guiding influence of the ultra-structural pattern of the surrounding basic substance. No orientating effect of the electric current or of certain tissue extracts was observed in vitro. Because the more recent in-situ experiments of WEISS and CAMPBELL (1944) showed no constant relation between the number of fibres of both stumps, they assumed a contrary attitude to the idea of chemotropism.

Quite antagonistic views regarding a guiding influence have been expressed. v. MURALT (1946) pointed out that certain observations concerning the degeneration phenomena of the distal stump support the idea of the emanation of specific substances which very probably attract the nerve sprouts. The absorption of fibres which have made unsuitable connections also pointed in the same direction. JENT and others (1945) have found a nerve extract with a proved speeding effect upon sensory fibres. LOVATT EVANS (1945), for example, said in his text-book that fuller recovery is gained when the stumps are not brought together for a week or two. The idea of an "Extrinsic Factor" with orientation and speeding effects upon nerve regeneration was first suggested by CAJAL in 1892. He supposed that neurotropism is analogous to chemical tissue activities. FORSSMAN'S (1898, 1900) experiments on the rabbit have had a great influence upon the development of the theory of chemotropism. In spite of criticism they have recently again aroused considerable attention (v. MURALT).

Clinical experience concerning nerve sutures shows that anatomical factors also influence the result. There are not many signs of an effective directive mechanism. According to MASSIE (1937) the suture of the musculo-spiral nerve is much more often successful than is the case with other upper extremity nerves, because an unavoidable derangement is minimized when the nerve is preponderatingly motor or sensory. This opinion is confirmed by the excellent results attained by BUNNELL (1944) in sutures of the purely sensory digital nerves. PLATT (1943) recommended the preservation of the intraneural topography when possible. SEDDON mentioned an intraneural plexus formation often a few centimeters proximal of the origin of a nerve branch. In an area of rearranged bundles an injury followed by a resection and suture is almost certain to bring together surfaces at which there is only imperfect funicular correspondence.

My aim during the last two and a half years has been to find out morphological signs of an orientation mechanism under simplified experimental conditions. The regeneration of motor and sensory fibres combined, or mostly each separately, after cutting and suturing the sciatic nerve has been examined histologically after varying times both in this nerve and in regard to one branch of it.

By observing the behaviour of both classes of regenerated fibres towards a sensory branch the study of signs of a guiding mechanism is possible without the injurious effect of a more direct experimentation technique on the very sensitive nervous tissue. This experimental method has not been used before, as far as I know, in research on nerve regeneration.

### Methods.

In all 29 full-grown cats were used. The operations were done under ether according to the open method with a premedication of 0.125 gr. avertin per body weight. To eliminate either the motor or sensory fibres the anterior or posterior roots were cut in alternative experiments. I tried to make a lasting denervation. An adequate sensory denervation is the more delicate of the preliminary operations. After a laminectomy from L4 to S1 four roots from L5 to S1 were regularly cut intradurally and the adjacent ganglia removed. They were usually easily recognizable. Occasionally the procedure was confirmed microscopically. In several experiments also the next higher and lower roots were cut, the number of roots sectioned together with the ganglia thus being six. Analogously 4 to 6 motor roots were cut.

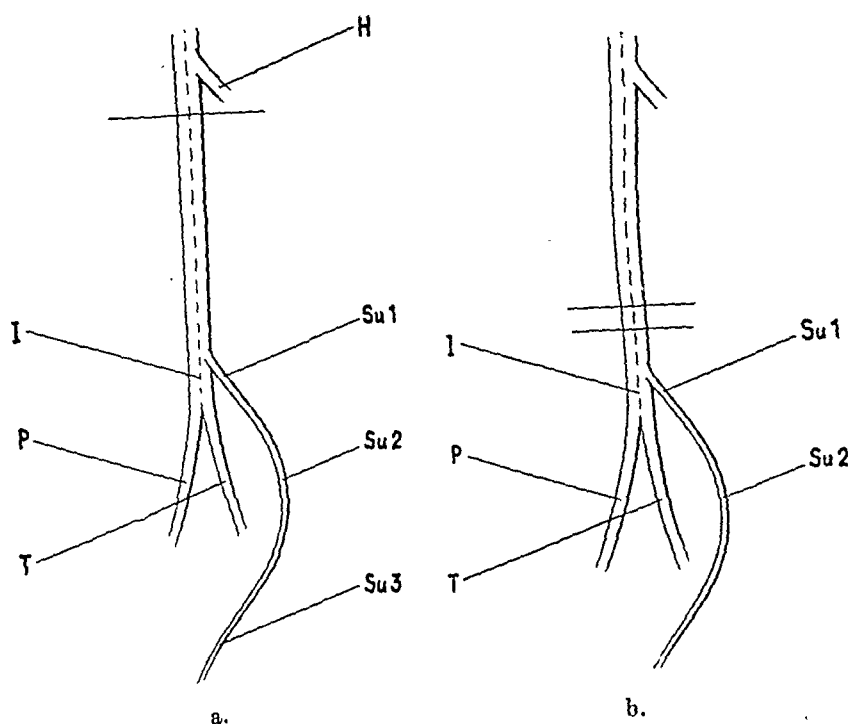


Fig. 1. a. Diagram of the nerves showing the level of cutting and suturing (horizontal line) and some typical levels of observation used in the majority of the experiments. H, hamstring branch. Su 1 — Su 3, levels of the suralis. I, T and P, levels of the sciatic, tibial and peroneal nerves. b. Diagram showing a variant of the technique used in animal 13.

In dealing with the sciatic nerve also a neuro-surgical technique was employed. A wider exposure of the nerve, especially in the uppermost part of it, was avoided; therefore the arterial blood supply from above which joins the nerve in the gluteal region of the cat (BENTLEY and SCHLAPP, 1943) was always left intact. In some experiments the sciatic nerve was cut at the same stage as the rhizotomy. A few millimeters' resection and the suturation was done 1—3 weeks later (Fig. 1, b). Mostly, however, the nerve was cut (Fig. 1, a) after application of indention sutures and sutured at a second operation 3—5 weeks after sectioning the roots when the condition of the animal had so much improved that further operations could safely be done. The nerve ends were joined by means of suturing with woman's hair recommended by GUTTMANN (1943) together with a few sutures of the finest available white silk.

I lost four animals in complications during the operations or a short time after it and five cats after a few days. All these operations with one exception pertained to the cutting of the posterior roots. — For the purpose of histological examination large specimens of normal, degenerated and reinnervated nerves were removed from anesthetized cats. In connection with this the action potentials in nerves with a preliminary motor denervation were recorded in situ or in a nerve chamber.



*Histological methods.* I have made extensive use of Flemming's solution for fixation and Wolter's modification (with Kultschitzky's haematoxylin solution) of the Weigert staining method for myelin sheaths.<sup>1</sup> This method has also been used by GUTMANN and SANDERS (1943). The fixative had the usual composition. In an earlier stage the nerve specimens were extended on cards but later on small pieces not exceeding a few millimeters were fixed for a couple of days. Also 10 % formalin was tried but found in general not suitable. The directions given by MALLORY (1938) in his manual were followed in the staining. The Davenport axon staining was made to some extent according to the descriptions of ROMEIS (1928) and WEIL (1944).

Difficulties in obtaining satisfactory stained sections by the Wolter method are mentioned in the literature. In this respect also I had many failures, especially in the beginning. Two more experiments are excluded for this reason. As the shrinkage connected especially with the fixation seemed not to be uniform, I do not give the micron values obtained in an extensive use of the ocular micrometer. The measurements are obviously of some value in comparing fibre diameters in different parts of the same specimen. The shrinkage does not interfere with the counting of the total number of suralis nerve fibres. The counting technique was modified from the methods used by GUTMANN and SANDERS, SIMPSON and YOUNG (1945) and others. After the transverse section of the suralis had been photographed, enlarged 300 times, clearly visible myelinated fibres in eight 1 centimeter squares were counted and the mean value was multiplied by the area of the nerve section.

Sections were mostly cut from comparable, easily recognizable levels (Fig. 1, a and b). The following method was used to make sure that a typical level of observation (Fig. 1, a and b, I) was included in the transverse sections. The branching point of the suralis in the popliteal space was set free by using neuro-surgical scissors. The nerve usually leaves the tibial portion and is directed from its dorsal and medial surface dorsally. A few millimeters of the sciatic nerve to which was attached about 5 mm. of the suralis was cut and embedded in a downward position.

## Experimental Findings.

For the purpose of describing the histological findings the experiments may be grouped as follows:

### I. Degeneration Experiments.

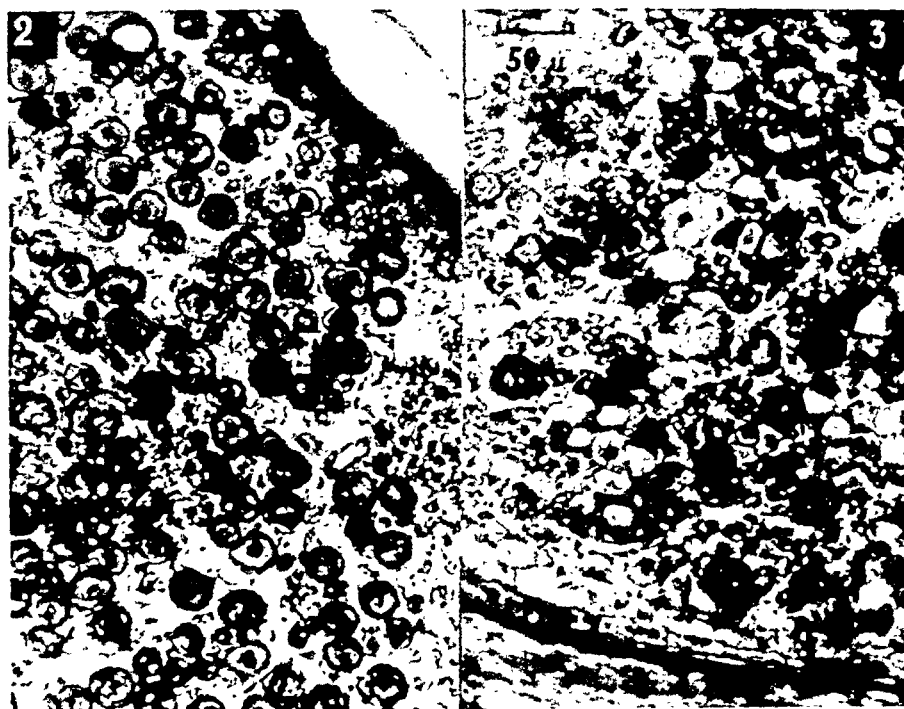
In man the suralis nerve which is formed from a branch of the tibial and the peroneal nerves is described as a typical super-

<sup>1</sup> The slides were made at the Pathological Institute of the University of Helsinki, Director: Prof. ARNO SAXÉN, M. D.

ficial nerve of the calf. The same is true of the cat in regard to its location and main fibre composition. As no definite information is available of running of motor fibres in the beginning of this nerve I have used the degeneration experiment to solve this question. Parts of the suralis of five cats three to six weeks after denervation operations were submitted to histological examination. In three of these cats a sensory denervation and in two a motor denervation of the sciatic nerve had been done.

Transverse sections stained by the Weigert-Wolter method show a fragmentation and a disappearance of the myelin sheaths as typical signs of degeneration after sensory denervation. After anterior root section, on the other hand, there is the usual picture of the nerve filled with well-marked transversally cut myelin sheaths. In animal 22 with a sensory denervation also the beginning of the suralis was thoroughly examined. In some sections a small number of well-stained myelinated fibres with about the same diameter as in the tibial nerve and some small fibres are to be seen. These observations are too few to allow any definite conclusions, but it seems probable that some motor and sympathetic motor fibres can leave a mixed nerve by using the root of a sensory nerve. — In summarizing the results derived of the first set of my experiments it can be stated that *the suralis nerve of the cat does not contain motor fibres except probably the first few millimeters.*

With the intention of getting the cross-section pattern of the sciatic nerve in regard to either motor or sensory fibres a number of sections were studied. They appertain to a nerve with respectively a sensory and a motor denervation each removed after 6 weeks degeneration. The peculiar aspect of the large diameter motor fibres in the tibial portion of the sciatic nerve of the thigh from animal 22 is noted. The number of fibres is not very large. At I level there is a clear grouping tendency. The largest number of fibres is to be found in the ventral and medial parts of the nerve (Figs. 2—3). Near the dorsal and lateral surface there are only very few motor fibres (Figs. 2—3) and also few small fibres accompanying them. A somewhat different pattern is seen higher up, and below the hamstring branch the motor fibres are almost diffusely distributed. The relatively large number of degenerated fibres and a comparison with the normal "crowded" transverse pattern of the nerve show that a purely conjunctional appearance of fibres is not probable. Even in the parts where motor fibres



Figs. 2—3. (Animal 22.) Motor fibres from ventro-medial and dorso-lateral parts of the tibial portion of the sciatic nerve after sensory denervation. Degeneration time 6 weeks. FLEMING, WEIGERT-WOLTER.  $\times 300$ .

are closer together (Fig. 2), there are sensory fibres around them. In sections from T level some 25 mm. below the branching-point of the suralis the tibial portion is divided into bundles without signs of grouping. The intraneural topography thus changes considerably at different levels. The peroneal portion has its own arrangement of fibres.

The sectioning of the anterior roots results in another pattern. The number of fibres in the sciatic nerve is now larger and the diameter even of the largest is smaller, about  $3/4$  of the motor fibres. The sensory fibres with a fairly constant size together with some small fibres are diffusely distributed all over the nerve with only small more or less empty spaces between the fibres as a result of degeneration.

The typical arrangement of the motor fibres especially at a certain level seems to be easily recognizable and it is of great interest to compare the corresponding transverse sections after regeneration in a sciatic nerve with a sensory denervation.

## II. Regeneration in Experiments without a Preliminary Denervation.

At the level used in the majority of the experiments, some 5 mm. below the hamstring branch (Fig. 1, a), the sciatic nerve in two cats was cut and immediately sutured. The animals were killed after 42 and 168 days.

A typical picture of a good regeneration is obtained. Myelinated fibres with varying diameters satisfactionarily matured, fill the section. The suralis is also filled by myelinated fibres which, however, have a more constant size. The diameter of the largest fibres is  $2/3$  of the normal. The number of the fibres at Su 2 level is 666. In the transverse sections of the 42 days' experiment stained by the Weigert-Wolter method, except for a few old degenerated fibres 1,332 very small fibres are seen. A high-power magnification shows these fibres to be thin-walled, often flattened transversally cut myelin sheaths. The number of fibres, *i. e.*, myelin sheaths in the suralis at a comparable level in the intact opposite limb of a cat previously operated and in the normal animal 29 is 528—544 (Fig. 4). Counting at Su 1 level gives about the same figures.

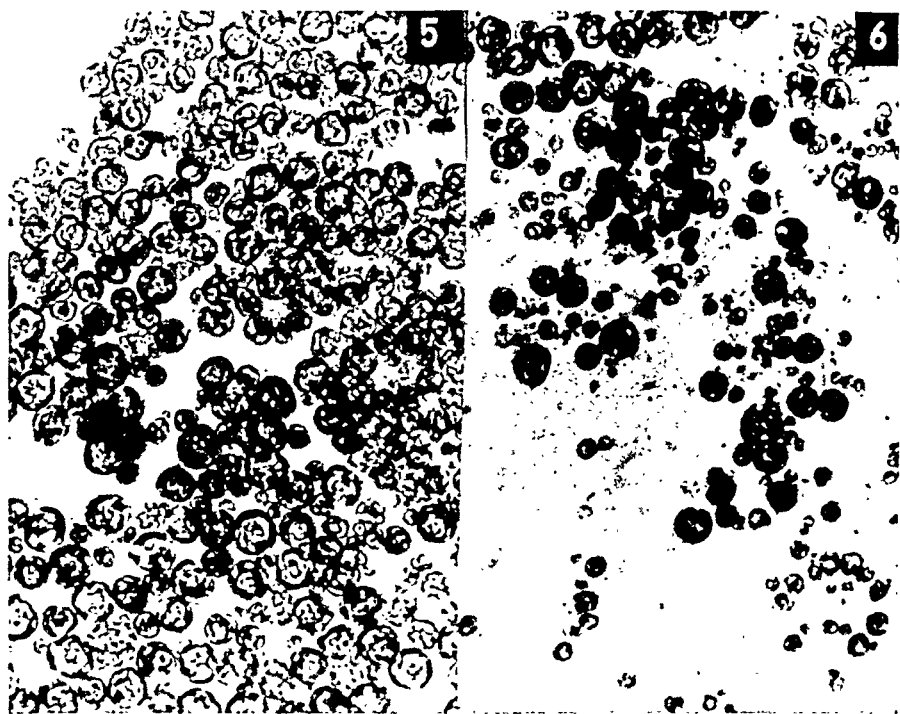
## III. Regeneration of the Motor Fibres.

In three experiments the sectioning of the sciatic nerve was done at the usual level. A previous sensory denervation had been carried out. In animal 13 (and 3 others) a variant of the technique was used (Fig. 1, b). The regeneration times were 21, 29, 70, 168 (2 experiments), 217 and 224 days.

In sections stained by the Weigert-Wolter method from specimens removed three weeks after a secondary suture only



Fig. 4. Transverse section of suralis fibres at Su 2 level from an intact opposite limb. FLEMING, WEIGERT-WOLTER.  $\times 300$ .



Figs. 5—6. (Animal 13.) Regenerated motor fibres from ventro-medial and dorso-lateral parts of the tibial portion of the sciatic nerve. Regeneration time 217 days. Figs. 2—3 and 5—6 are comparable transverse sections. FLEMING, WEIGERT-WOLTER.  $\times 300$ .

the voluminous Schwann cells appear clearly. No myelinated fibres are found. In another cat after four weeks, however, a considerable number of small very thinwalled transversally cut myelin sheaths with an irregular wave-like appearance here and there, are seen. Almost the same picture is obtained from the 70 days' experiment. In the late stages mostly a good maturation had been reached, the fibre diameter occasionally (animal 13) remaining only very little below the normal. In this experiment the suture of the sciatic nerve had been done after a 3 weeks predegeneration. A comparable experiment with the suturation 1 week after the cutting of the sciatic nerve shows a less satisfactory regeneration. Sections obtained from I level often — especially in animal 13 (Figs. 5—6) — show a clear grouping tendency of the regenerated fibres. In one or two cats it was not recognizable because the nerve had already at the level of examination split into bundles.

Transverse sections from the suralis nerve were made from four cats after 70 to 217 days. After the shortest time the number of the fibres found at Su 2 level is 423. At Su 2 and Su 3 levels

in two instances (animals 16 and 26) after 169 days about 38—60 small groups of fibres or separate fibres can be counted (Fig. 7). There is no great difference in the growth of equal-sized fibres into the first millimeters of the suralis. Because the course of the fibres is more irregular than usual and consequently only a few transverse cuts appear, it is not easy to obtain the exact number but an approximate one is 70. The diameter of the fibres corresponds with that of small, probably sympathetic fibres in the normal nerve. Around the fibres small collections of very thin non-myelinated fibres are made visible by the Davenport axon staining. — Longitudinal sections of the nerve union show mostly after a previous motor denervation a satisfactory connection (Fig. 8). In late stages it is not possible to see the gap between the stumps. Occasionally a number of fibres in the epineurium is to be seen. In one case the fibres run directly towards the other visible end.



Fig. 7. (Animal 26.) Previous sensory denervation. Transverse section of the suralis at Su 2 level. Only very few small fibres are present after 168 days. FLEMING, WEIGERT-WOLTER.  $\times 300$ .

The histological findings in animal 13 after 217 days regeneration are somewhat exceptional. The growth has occurred uniformly at Su 1 and Su 2 levels. The number of the fibres, many of which are larger than regenerated suralis fibres in other instances, is 127 (Fig. 9).

#### IV. Regeneration of the Sensory Fibres.

The sciatic nerve with a previous motor denervation was cut and sutured as usual. The animals were examined after 168 (2 experiments) and 217 days.

A good regeneration with diffusely distributed fibres has occurred. The diameter of the largest myelinated fibres is about half



Fig. 8. (Animal 16.) Longitudinal section of the union of the sciatic nerve with a previous motor denervation showing the growth of the sensory fibres through the gap. Regeneration time 168 days. The central stump is above. FLEMING, WEIGERT-WOLTER.  $\times 80$ .

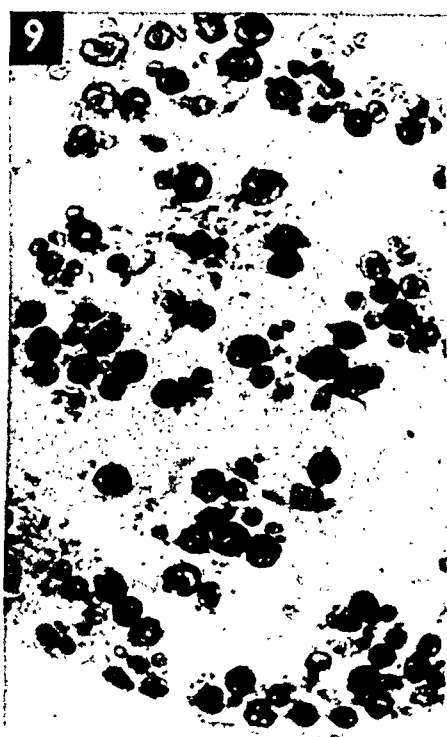


Fig. 9. (Animal 13.) Previous sensory denervation. Transverse section of the suralis at Su 2 level after 217 days. Note the diameter of the fibres when compared with Fig. 4 and Figs. 5-6. FLEMING, WEIGERT-WOLTER.  $\times 300$ .

the normal. — The suralis growth at different levels is uniform. Counting gives the figures 528, 539, and 708 at Su 2 level. The fibre diameter which in the six months experiments (Fig. 10) is much below the normal is somewhat larger in the experiment lasting about  $1\frac{1}{2}$  months longer.

*Functional factors.* The return of the muscle function in the distal part of the hind limb was followed in cats without a preliminary denervation and in the experiments concerning the regeneration of motor fibres. Observations in 7 experiments of longer duration show consistently that the first settled signs of movements in the extensor group below the knee appear after 8 weeks and occasionally even sooner. They were followed by more or less progress also on the flexor side. Dorsiflexion of the toes never returned. — An attempt was made to get an idea of the reappear-

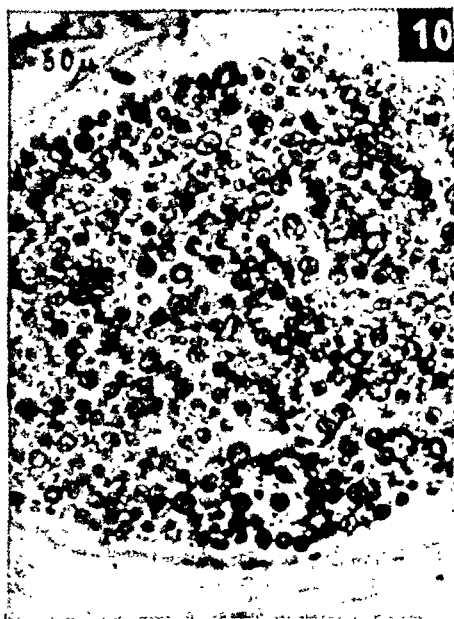


Fig. 10. (Animal 19.) Previous motor denervation. Transverse section of the suralis at Su 2 level after 168 days. FLEMMING, WEIGERT-WOLTER.  $\times 300$ .

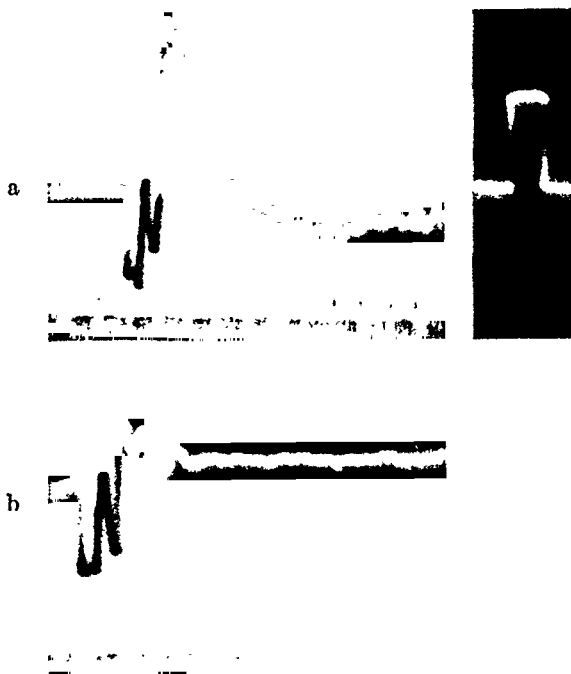


Fig. 11. (Animal 19.) a. Action potential set up in tibial and peroneal nerves together. b. Action potential almost extinguished after crushing of the sciatic nerve. Calibration with 1 mV for a and b. Time, 0.01 sec.

ance of sensibility by pricking and pinching the skin of the extremity. This is, however, a very ambiguous method in animals.

For this reason the conduction properties of nerves with regenerated sensory fibres were to some extent studied electrophysiologically.<sup>1</sup> In three cats after 28—70 days there were no spike potentials and no after-potentials passing through the suture line. The same is true in regard of the suralis after sensory denervation in one instance. In one experiment the action currents in the suralis trunk and in the tibial and peroneal nerves with satisfactory regeneration of the sensory fibres were recorded. The other electrode isolated from the muscles had been placed above the union at the upper end of the sciatic nerve. The sciatic nerve had been divided in the sciatic notch at the beginning of the examination. A conductivity was shown by the recording (Fig. 11). There was naturally a difference in the magnitude of the potentials de-

<sup>1</sup> The recording of the action potentials was done by P.-O. THERMAN, M. D. G. SVAETICHIN, L. M., and Y. TEMMES, L. M.



pending on the different fibre number of the nerves considered. Satisfactory myelination thus is an essential criterion of reestablished conduction ability.

### Discussion.

The number of my experiments successfully carried out is too small to give any definite account. In addition to that they pertain mostly to the late stages of regeneration. Some constant results were, however, obtained from a number of comparable experiments. My observations are also confirmed by the work of other researchers.

SANDERS and YOUNG (1944) have observed that in the lower part of the thigh of the rabbit the normal peroneal and tibial nerves divide into two sets of funiculi each containing motor and predominantly sensory fibres. My results show that under favourable conditions the reappearance of the regenerated motor fibres in normal groups can be demonstrated.

Earlier and more recent cross-union experiments give some idea of the possibilities of a certain class of nerve fibres growing into a nerve with the same or a different kind of fibres. According to LANGLEY and ANDERSON (1904) the attraction exerted by the cut peripheral end is greater in the motor fibres and in the sympathetic fibres among themselves than the attraction of the fibres of the two classes to each other. The fibres may grow some distance even in an unsuitable distal end probably owing to the nutritional value of the peripheral nerve. Other experiments have obtained functional connections which earlier were considered impossible. SIMPSON and YOUNG joined the central end of a spinal nerve to a postganglionic sympathetic nerve. This resulted in a regeneration below the union line with very small myelinated fibres numbering about half the normal. — In the experiments dealt with above the fibres are forced to grow in a certain direction, the only choice being the joined peripheral end. In my experiments again the growth of the motor or sensory fibres has the possibility of directing either towards an appropriate or an inappropriate Schwann tube.

In two experiments with a previous sensory denervation and the usual level of cutting and suturing no regenerated fibres with motor fibre diameter had grown into the sensory branch after 168 days. In three comparative experiments with the inverse

technique fibres whose functional properties were occasionally studied electrophysiologically grew into the suralis nerve. The examination of morphological signs alone in the nerve regeneration is not enough. For example number of the fibres is not a sufficient criterion of good regeneration because SIMPSON and YOUNG have observed a more numerous growth of smaller fibres when the end-organs could not be reached.

As in the 70 days' experiment the number of fibres was considerable it has not been proved that motor fibres in an early stage of regeneration should reject a sensory branch. If the majority of the young fibres are motor, which is very likely, it seems not possible to understand the absence of these fibres in a late stage without the principle of absorption. This is — except the re-appearance of group formation — the only certain sign of an orientation mechanism which was found.

Unfortunately there is no adequate electrophysiological confirmation of the uniformly regenerated suralis fibres in animal 13 but their exceptional size makes it possible that they are motor fibres. It is to be borne in mind that sensory fibres were absent. As it seems probable that fibres which have made unsuitable connections are not always absorbed, the idea of a central reorganization emphasized by WEISS, WRIGHT (1945) and others may prove valid.

There was no change in the number of fibres in the suralis after regeneration in the experiments with 3—5 weeks predegeneration of the motor fibres of the sciatic nerve. Therefore it seems probable that this form of degeneration has no attractive effect. According to the histological study the appearance of Schwann cells with a voluminous cytoplasm and great nuclei was lacking, contrary to the degeneration which is followed by a regeneration. FORSSMAN earlier pointed out that not every kind of degenerated nerve tissue possesses a chemotrophic activity.

The small fibres in the suralis which appear to some extent in spite of a sensory denervation of the sciatic nerve are probably sympathetic fibres whose great regenerating power is recently pointed out by BARCROFT and HAMILTON (1948) among others. The small non-myelinated fibres may be sympathetic vasodilatator fibres whose existence is presupposed by WHITE and SMITHWICK (1944) and others. Their cell bodies could have been in the small parts left of dorsal root ganglia or in the nerve trunk on the distal side. Trophic changes which almost always were

connected with the sensory denervation disappeared later when sympathetic fibre regeneration had had time to arise.

### Summary.

1. The regeneration of motor or sensory fibres after cutting and suturing the sciatic nerve was examined histologically. By previous denervation one kind of fibre was eliminated in each set of operation. By observing the behaviour of both classes of regenerated fibres towards a sensory branch (suralis) with a confirmed normal composition morphological signs of an orientation mechanism can be studied without the injurious effect of a more direct experimentation technique.

2. The reappearance of the regenerated motor fibres in a normal group-formation in the tibial portion of the sciatic nerve may be recognized at a comparable level.

3. A normal fibre number, a uniform growth and an electrophysiologically confirmed conductivity in the suralis nerve was obtained only when sensory fibres were available in the sciatic nerve. In two cats with a previous sensory denervation of the sciatic nerve only a very limited number of small fibres in the suralis was in a late stage found. In an experiment with a shorter duration the number of fibres was somewhat larger. Consequently there must be an absorption of fibres which have grown into an unsuitable branch.

4. There were no signs of an attractive effect combined with fibre degeneration which was not followed by a regeneration of the same class of fibres.

5. An abnormal growth of a number of what were probably motor fibres into the suralis was connected with the cutting and suturing of the sciatic nerve with a previous sensory denervation a few millimeters above the branching point of the suralis. It is probable that fibres which have made unsuitable connections are not always absorbed. Therefore also the idea of a central reorganization may prove valid.

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# **The Total Quantity of Hemoglobin in Man and its Relation to Age, Sex, Bodyweight and Height.**

By

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In a previous work (SJÖSTRAND 1948) in connection with the description of a method for determining the total quantity of hemoglobin, some values have been stated showing a considerable difference between the two sexes and even between different individuals of the same sex. It seems interesting to study this variability more closely, and in the first place to endeavour to elucidate the total quantity of hemoglobin with relation to age, bodyweight and height.

## **Method and Material.**

The total quantity of hemoglobin in the body was determined according to SJÖSTRAND (1948). Double determinations have been carried out to a great extent, and if they have shown a greater deviation than 8 %, then yet another and possibly several other determinations have been made.

The relative Hb value has been determined in "capillary" blood from a finger and in a Sicca-Hemometer; 100 % corresponding to 15.4 gram hemoglobin.

The adults examined consisted of nursing staff, laboratory assistants and patients, who have visited the medical out door clinic of the Caroline Hospital, but were not found to be suffering from heart or blood diseases or any other complaint that could be expected to have any effect on the total quantity of hemoglobin. The children have been

Table 1.

*Data Concerning the Material Investigated.*

	Number	Average age years	Age limit	Average weight Kg	Weight limit	Average height Cm	Limit for height
Girls....	21	13.9 $\pm$ 0.5	8—16	42.1 $\pm$ 2.6	29—69	147.9 $\pm$ 2.7	129—171
Boys ...	17	14.7 $\pm$ 0.5	8—17	41.7 $\pm$ 2.7	29—70	150.8 $\pm$ 3.0	129—173
Women.	92	37.6 $\pm$ 1.3	17—70	65.5 $\pm$ 1.3	41—108	160.0 $\pm$ 1.0	149—179
Men ....	174	23.9 $\pm$ 0.6	18—57	69.7 $\pm$ 0.6	53—98	176.7 $\pm$ 0.5	160—197

chosen from an elementary school and have been found to be healthy after a general medical examination.

Table 1 gives some statements concerning the material.

### Results.

*The Quantity of Hemoglobin in Relation to Age.* Fig. 1 illustrates the correlation between the total quantity of hemoglobin and the age for all the material examined, which has been divided up according to sex. The dots in the figure show the average calculated usually from at least ten determinations. As will be seen from the distribution of the dots, the scope of the age varies for the different calculations, which has been done on purpose to reduce the individual spread of the values.

From fig. 1 it will be seen that the quantity of hemoglobin increases fairly evenly during the years of development. There seems to be no definite difference between the sexes up to puberty, but between the ages of 12 and 13 the curve deviates for the girls, the increase of hemoglobin in their case decreasing successively. At the age of 20 the quantity of hemoglobin seems to be constant in the female material. In the case of the males the quantity of hemoglobin increases fairly regularly with the age up to 18, after which the increase becomes less, and at 22—23 the quantity of hemoglobin seems to be constant.

Since the material for males comprises comparatively few individuals over 35 years of age, a reservation must be made for the higher ages. The material for women is more evenly distributed in respect to age, in consequence of which the curve may be considered fairly representative up to the age of 55.

*The Quantity of Hemoglobin in Relation to Sex.* As will be seen from fig. 1, there is little or no difference between the two sexes as regards the hemoglobin between the ages of 8 and 12. There

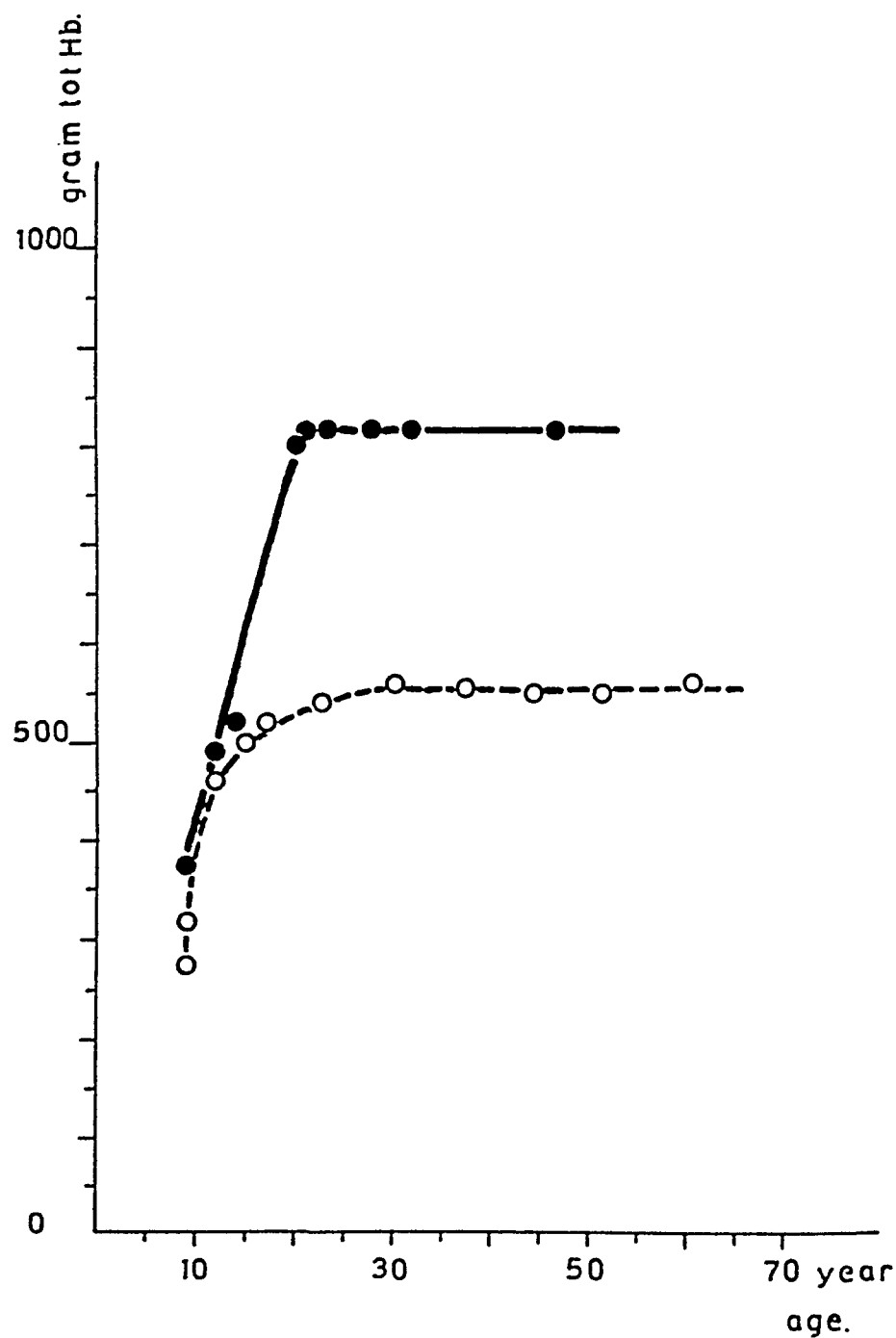


Table 2.

*Correlation of Total Quantity of Hemoglobin with Bodyweight.*

	Total Hb gram	Total Hb % of bodyweight	Correlation Total Hb/bodyweight
Girls.....	411 $\pm$ 28	0.97 $\pm$ 0.035	0.87 $\pm$ 0.05
Boys .....	471 $\pm$ 33	1.13 $\pm$ 0.033	0.91 $\pm$ 0.04
Women .....	554 $\pm$ 11	0.86 $\pm$ 0.013	0.67 $\pm$ 0.06
Men .....	803 $\pm$ 8	1.16 $\pm$ 0.01	0.56 $\pm$ 0.05

is, however, on the other hand, a considerable differentiation in the sexes at and after the age of puberty. Thus, in the case of the adult woman, the total quantity of hemoglobin is about 30 % under the hemoglobin value of the man, the average for the quantity of hemoglobin for the man being  $803 \pm 8$  grammes and for the woman  $555 \pm 11$  grammes.

*The Quantity of Hemoglobin in Relation to Bodyweight.* In consequence of the age and sex variations in connection with the quantity of hemoglobin, the material has been divided up into four groups: girls, boys, women and men, and the correlation coefficient for the relation of the quantity of hemoglobin to bodyweight has been calculated for these four groups separately. Table 2 shows that there is a good correlation between the quantity of hemoglobin and the bodyweight of children, the correlation being considerably less in women and fairly poor in men. The quantity of hemoglobin in percentage of bodyweight is greatest in men and least in women. Even before the puberty there seems to be a similar differentiation. The difference in the quantity of hemoglobin in man and woman in relation to the bodyweight is on an average 25 %, calculated on the value of man. Table 2 also shows that the individual variability of the total Hb value reckoned on the bodyweight is rather great. The standard deviation varies between 12 and 16 % on the material examined.

Fig. 2 illustrates the relation between the quantity of hemoglobin as per cent of bodyweight and the age of the two sexes as well as the relation of the relative Hb value to the age. The figure shows that the procentual total hemoglobin value produces rather considerable age variations, which is not obvious from the curves of the absolute total hemoglobin values for the different ages (fig. 1). From this it is evident that the total quantity of hemoglobin does not seem to be directly correlated to the bodyweight. During the earlier years of development, the difference — which



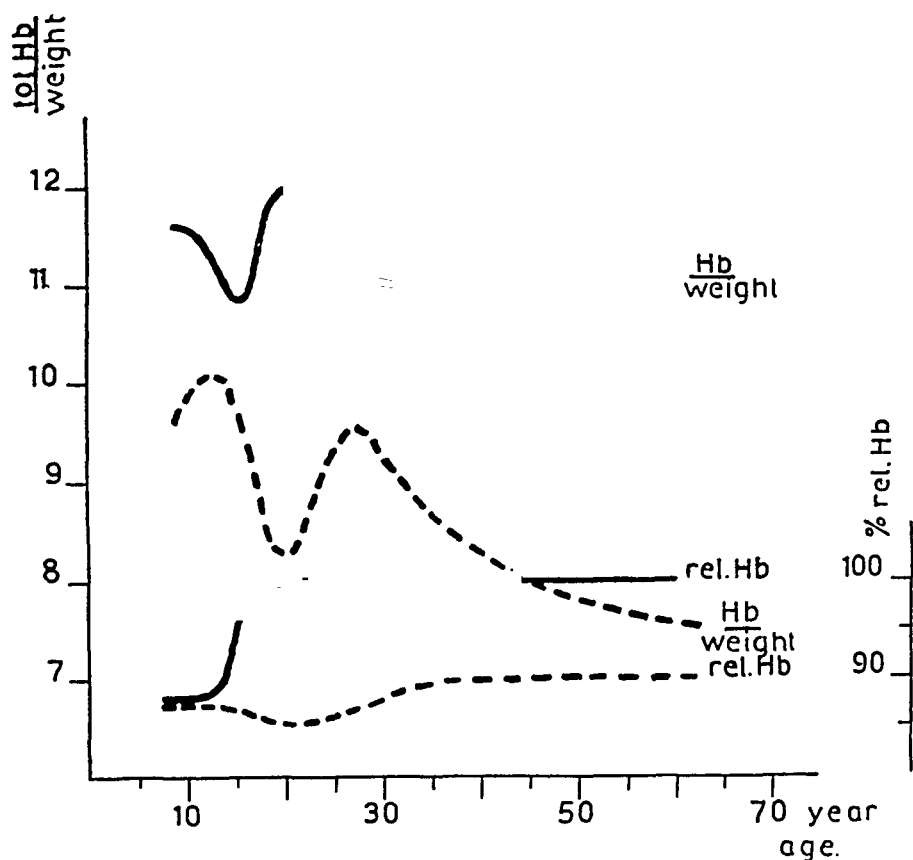


Fig. 2. The relation of  $\frac{\text{total Hb}}{\text{bodyweight}}$  and relative Hb to age on males (continuous lines) and females (dashed lines).

has already been pointed out — seems to lie in the fact that in comparison to the bodyweight boys have a greater quantity of hemoglobin than girls (1.17 % of the bodyweight of boys between the ages of 8 and 12, as compared with 0.96 % of girls of the same age). This difference seems to increase still more in connection with the hormonal evolution during the age of puberty. Thus, round about 15 years of age the procentual quantity of hemoglobin increases considerably in boys, while it decreases in the case of girls somewhat earlier, about the age of 12—13. After the age of 20 the two curves again show a tendency to a certain levelling of the difference in sex, though at about 30 the procentual total Hb value again declines in the female material. Taken as an outline these differences seem to be significant, in spite of the quantity of material examined being fairly small especially in respect to ages before the puberty.

This difference in sex also stands out in the relative Hb value, which increases round about an age of 13 in the case of boys, but decreases somewhat in girls after this age and continues to do so up to the age of 35.

These differences with regard to sex may be best explained by the fact that to the zygomatic sex differentiation concerning the total Hb value in relation to the bodyweight, there appears in connection with puberty a differentiation conditioned by the sexual hormones. Thus the secondary sexual development in the male implies an increase of the blood formation relative to bodyweight, whereas it has an opposite effect on the female.

The decrease in the quantity of hemoglobin in relation to the bodyweight of the female material after the age of 30 seems, on the other hand, to be allied to an increase in the bodyweight, which is not accompanied by any increase in height, as will be seen from fig. 3, where the curves are found for the weight, the height and the total Hb values for the material examined. This increase in weight is probably caused either entirely or largely by an increase in the adipose tissue, and accordingly there is no corresponding increase in the quantity of hemoglobin. In the male material there is no similar decrease in the total quantity of hemoglobin in per cent of the bodyweight, despite the fact that this material, too, shows a certain increase in bodyweight and instead rather a decrease in height with age. As has already been pointed out, however, the material concerning ages over 30 is too insufficient to allow of any definite conclusion.

Accordingly these observations concerning the relation of the quantity of hemoglobin to the bodyweight seem to show:

- 1) that there is a difference between the two sexes previous to puberty,

- 2) that this difference increases with the evolution during puberty at which time the masculine hormonal change seems to imply stimulation of the relative blood formation, whereas the feminine change seems to signify an inhibition,

- 3) that an increase in the adipose tissue at least as far as the woman is concerned, does not occasion a corresponding increase in the quantity of hemoglobin.

Nevertheless, even if consideration is taken to this variability of the procentual total Hb value, the material examined shows a fairly appreciable individual variability.

*The Relation of the Quantity of Hemoglobin to Height.* From table

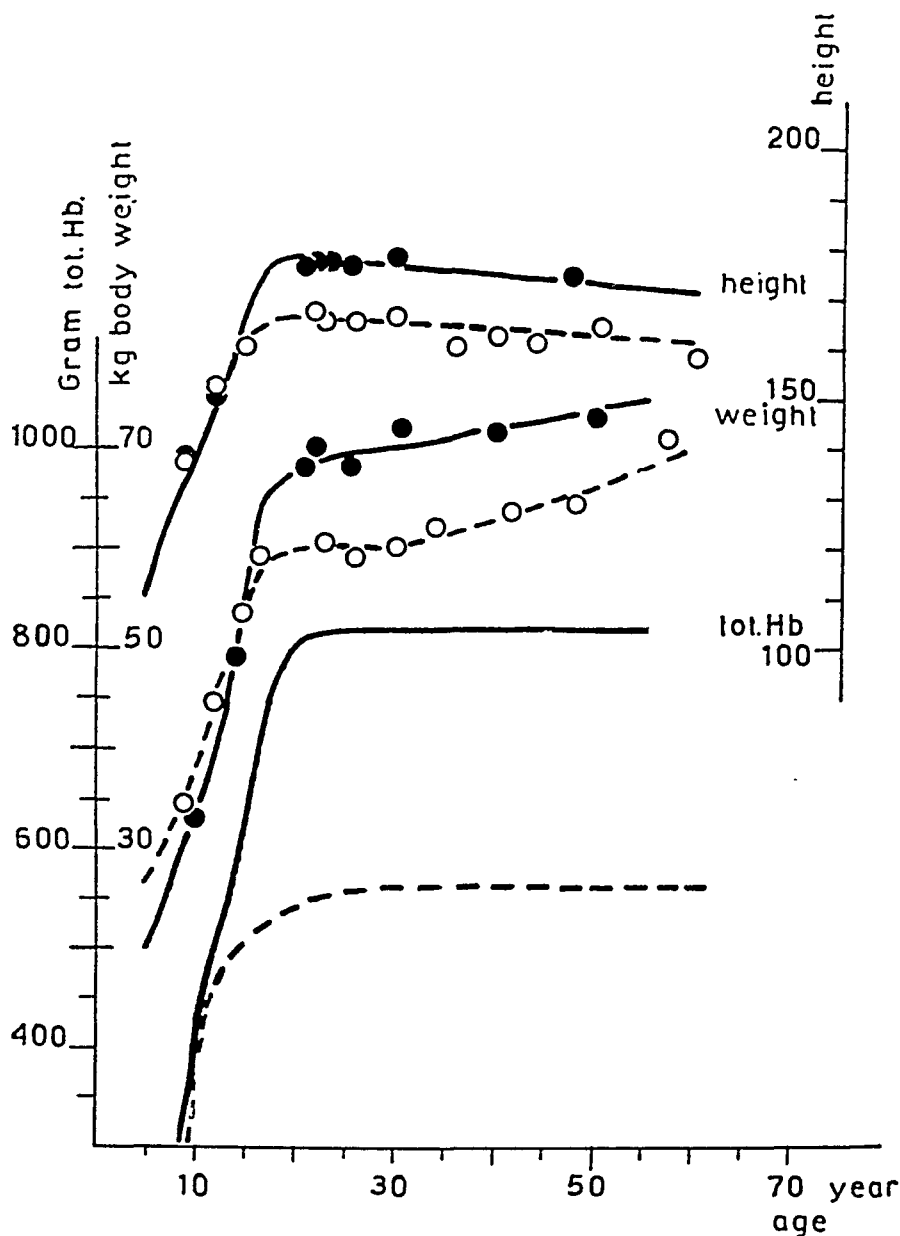


Fig. 3. Quantity of hemoglobin, bodyweight and height in relation to age on males and females.

3 it will be seen that correlation is good between the quantity of hemoglobin and height in the material of children and that it is very small in the material of adult; men as well as women.

Fig. 3 shows that the quantity of hemoglobin fairly well follows the curve for the height of the man. In the case of the woman,

Table 3.

*Correlation of Total Quantity of Hemoglobin with Height.*

	Correlation Total Hb/height.
Girls.....	0.86 $\pm$ 0.06
Boys .....	0.79 $\pm$ 0.09
Women .....	0.3 $\pm$ 0.1
Men .....	0.41 $\pm$ 0.06

Table 4.

*Correlation of Total Quantity of Hemoglobin with Bodysurface.*

	Total Hb gram/sq.m bodysurface	Correlation Total Hb/body-surface
Girls.....	308 $\pm$ 13	0.87 $\pm$ 0.05
Boys .....	350 $\pm$ 13	0.88 $\pm$ 0.06
Women .....	326 $\pm$ 5	0.69 $\pm$ 0.05
Men .....	431 $\pm$ 4	0.59 $\pm$ 0.05

however, the hemoglobin curve deviates from the one representing the height between the ages of 12 and 13.

*The Relation of the Quantity of Hemoglobin to the Surface Area of the Body.* Different factors use to be correlated, such as the basal metabolism, and the minute volume of the heart at rest and the volume of the heart, to the surface area of the body, and it may therefore be of interest also to correlate the quantity of hemoglobin to the body surface. Since the quantity of hemoglobin shows correlation both to bodyweight and height, the correlation to bodysurface may be expected to be higher than to bodyweight. Table 4 shows that this is also the case with both men and women, though not with children. The difference between the correlation coefficients is, however, only slight. In this case the surface area of the body has been calculated according to Du Bois' formula.

*The Relation between the Hemoglobin Quantity and the Blood Volume.* The blood volume can be calculated from the total hemoglobin and the relative hemoglobin values. Such a calculation, however, is not quite reliable, since the relative Hb value varies in different parts of the vascular system (SMITH, ARNOLD and WHIPPLE 1921, FÄHRÆUS 1929, HAHN, ROSS, BALE, BALFOUR and WHIPPLE 1942). Under the same test conditions, a calculation of the blood volume in this manner gives, however, a comparative

measure for different individuals. The results obtained on children before and during puberty is also comparable to those found by BRINES, GIBSON and KUNKEL (1941) by estimating the plasma volume.

By making a similar calculation of the blood volume of the material here investigated, it has been found that broadly speaking there is the same variability as in the total hemoglobin quantity. The difference in the blood volume between the two sexes is, however, about 10 % less than the difference between the hemoglobin quantities. The decrease found in the hemoglobin quantity in relation to the bodyweight, in case of women towards the end and after puberty up to the age of 30, corresponds to a seemingly somewhat less decrease in the blood volume.

### Discussion.

The values of the hemoglobin quantity found by means of the method used should be corrected as regards the error of the method which lies in the fact that the carbon monoxide is combined *inter alia* to the myoglobin. In a previous work (SJÖSTRAND 1948) this question came up for discussion and several factors were stated indicating theoretically that this error is slight. A comparison between the values of the total hemoglobin quantity found by means of this method, from which the weight of the blood corpuscles can be calculated, and the values of the blood corpuscle weight determined by other methods indicated in literature, also showed that the method does not seem to be at any disadvantage owing to greater systematic errors than the most reliable methods, in which carbon monoxide is not used. A more exact comparison between different methods in this manner is not, however, possible, since the statements so far published of comparable extensive material do not include data of the sex of the individuals, and very often not even the age, bodyweight and height.

There is yet another support in favour of the opinion that the myoglobin error is slight in determinations carried out according to the method adopted in this work, which support has been obtained by determining the total hemoglobin quantity in cases of anemia, followed by blood regeneration. In acute hemorrhages the absolute hemoglobin values are comparatively lower than the relative Hb values (see SJÖSTRAND 1948), owing to the plasma

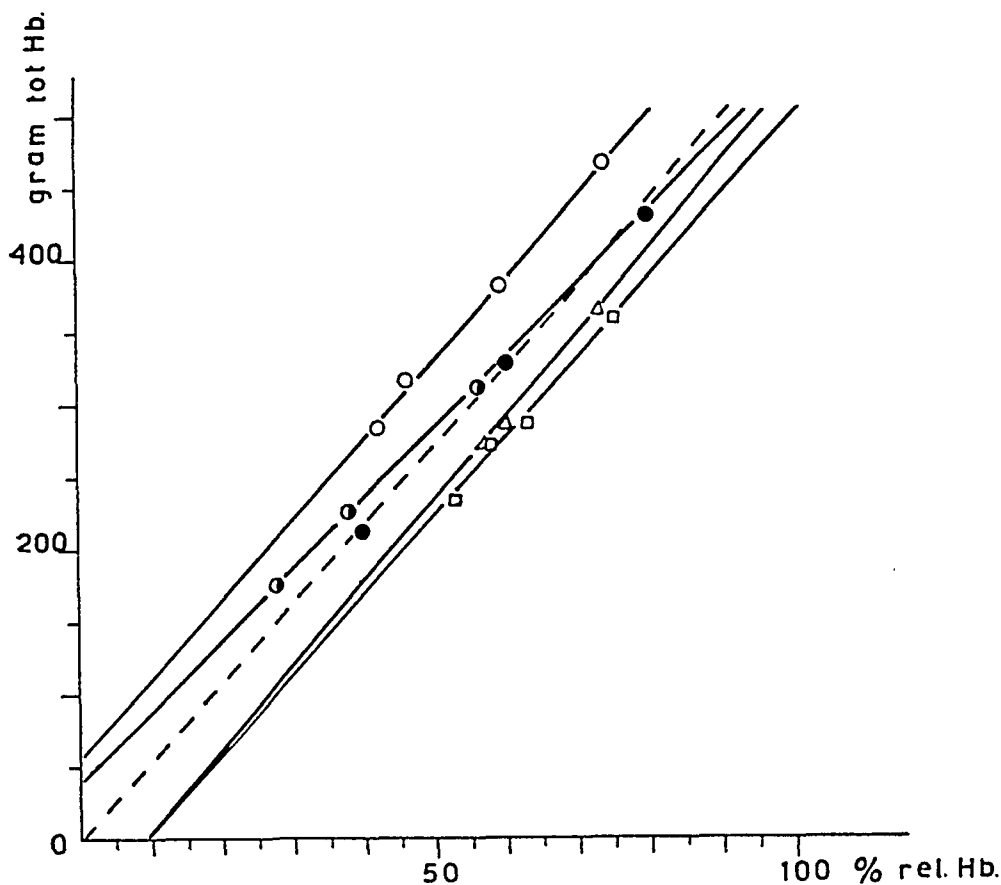


Fig. 4. The relation of quantity of hemoglobin to relative Hb on four cases of chronic anemia during recovery. The dashed line = mean curve.

volume not altogether compensating the loss of blood cells. In cases of chronic anemia, on the other hand, the relation may be expected to be different in this respect. This will be seen from fig. 4, which shows the correlation between the total hemoglobin quantities and the relative Hb values in four cases of chronic anemia, two of which were pernicious anemia, the third secondary anemia and the fourth anemia with chronic nephritis with nephrosis. If the lines of regression are lengthened, they will intersect the total Hb axis in the two pernicious cases, but in the two latter at the relative Hb axis and with about 10 % deviation from the zero point. The mean line, on the other hand, intersects almost exactly the zero point. This may naturally be just a coincidence, and the plasma volume may increase more than what corresponds to the decrease of hemoglobin in the pernicious

anemia cases. ROWNTREE and BROWN (1929) also contend that this is the case, though others dispute it. On the other hand the other two cases of anemia do not seem to have a full compensation for the loss of blood cells through the increase of the plasma volume. These observations, however, indicate that the myoglobin error is slight, as otherwise a systematic deviation of the mean line might be expected.

When carrying out repeated determinations with 15 minute tests of the alveolar CO concentration, the second test (completed 30 minutes after the carbon monoxide had been added) was on an average 5 % lower than might have been expected from the first 15 minute value and the continuous decrease of the following 15 minute values. The latter decrease corresponded almost entirely to the CO quantity which was eliminated as every test was taken. As was pointed out in the previous work, the 5 % decrease seems to be entirely or largely due to the carbon monoxide being taken up extravascularly. If it is assumed that the myoglobin absorbs the carbon monoxide at the same rate in relation to the CO partial pressure during the first 15 minutes, the CO quantity then absorbed should be about half of what is absorbed during the following 15 minutes, the CO partial pressure increasing fairly uniformly during the first test time from 0 to the pressure prevailing at the second 15 minute period. This would make the myoglobin error about 2.5 %, which seems to be in good agreement with determinations carried out by ROUGHTON and ROOT (1945) and ROOT, ROUGHTON and GREGERSEN (1946) on the CO elimination from the blood during one hour after the carbon monoxide has been added. From their results they reckon that 2 % or 2.5 % of the carbon monoxide added is absorbed extravascularly during a mixing time of 20 minutes for the blood volume determination.<sup>1</sup>

Accordingly the hemoglobin values thus obtained should be reduced by about 2 or 3 %. A similar reduction has not, however, been made in this work, partly on account of the uncertainty attached to the calculation of this reduction factor, partly because it has been considered more practical to compare the values found actually under the methodical conditions indicated.

<sup>1</sup> BJÖRCK (1949) gives some data about the relative affinity of myoglobin and hemoglobin. His dissociation curves are, however, hypothetical at the low CO pressure in question and, in addition, inaccurately drawn. Furthermore, he does not recognize, that the dissociation curves must be calculated at a comparable oxygen pressure. His conclusions are, consequently, invalid.

The age and sex variations of the hemoglobin quantity will be of interest in several respects. The apparently hormonal effect on the relative blood formation may be of importance as a cause of anemia in girls and young women. The relative decrease in the blood volume in relation to the height at the time of puberty in the woman may also be of significance in orthostatic disturbances of the blood circulation and also in the case of circulatory adaptation in connection with physical work and pathological conditions. These conditions as well as an explanation of some of the individual variability of the total hemoglobin quantity and the blood volume will be gone into more closely in subsequent papers.

### Summary.

According to a method already described the total quantity of hemoglobin has been determined on 17 boys and 21 girls between the ages of 8 and 17, on 174 men between the ages of 18 and 57 and on 92 women between 17 and 70.

In the case of the male material the total quantity of hemoglobin increased with age and broadly speaking with physical growth up to the age of 22. In the female material — as in the male — the quantity of hemoglobin increased up to the ages of 12 to 13, but after this, the increase was considerably less up to the age of 20, after which it remained constant.

In the male material the quantity of hemoglobin showed a manifest increase in relation to the bodyweight during the years of puberty and up to the age of 22, but in the female material there was a relative decrease from the age of 12 to 20.

The average quantity of hemoglobin in the adult man was 1.16 % of the bodyweight and 0.86 % in the adult woman.

Even previous to the years of puberty the sexes showed a difference concerning the total quantity of hemoglobin in relation to the bodyweight but none in relation to age.

The quantity of hemoglobin did not increase with the increase of weight after physical growth had ceased, at least as regards the female material.

The quantity of hemoglobin showed good correlation with the height during the years of development, though there was only slight correlation in the case of men and women. As regards the surface area of the body the total quantity of hemoglobin showed



only slightly better correlation than to the bodyweight in men and women, but it was less in children.

Broadly speaking the blood volume showed the same variability as the total quantity of hemoglobin, except that the difference between the sexes, in adults, was about 10 % lower as regards the blood volume than it was concerning the quantity of hemoglobin.

The size of the error which the method adopted has, in that part of the carbon monoxide is bound extravascularly inter alia to the myoglobin is discussed and the total hemoglobin determinations of some chronic cases of anemia are given as a further proof of the smallness of this error, which is reckoned as being from 2 to 3 %.

The significance of the seemingly hormonal effect on the hemoglobin production under and after puberty is also discussed.

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## The Food Sparing Effect of Liver Extracts on Rats.

By

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In a recent paper it was shown that the daily administration of about 0.2 g of a catheptic casein hydrolysate to young rats significantly increased the weight gain per g food eaten (ÅGREN 1947). The experimental group of animals, like the control group, was given a stock diet and water *ad libitum*. The average daily food consumption was  $11.0 \pm 0.24$  g in the experimental group and  $16.3 \pm 0.31$  g in the control group. However, the average daily weight gain was the same in the two groups of animals. This "food sparing" action of the extract was supposed to be correlated with the presence of growth-stimulating, enzyme resistant peptides in the digests. These factors have later been investigated in some details by means of microbiological and chromatographic methods (ÅGREN 1949, DE VERDIER and ÅGREN 1948).

From the nutritional point of view these results were of both theoretical and practical interest and a similar series of experiments was accordingly carried out using a liver extract as a potential source of factors with similar biological properties.

### Experimental.

The animals were young albino rats with an average weight of about 50 g. They were kept in individual cages. Water and the commercial mouse bread described by GARD (1944) were given *ad libitum*. The total nitrogen content of the liver concentrate was 17 mg per ml. Each ml

Table 1.

*Growth of rats on a mixed diet with the addition of a liver extract.*

Group	Liver extract in mg per day and animal	No. of animals	Average daily weight gain	Average daily food consumption	Weight gain per g food eaten
I.....	54	10	$2.97 \pm 0.04$	$8.72 \pm 0.25$	$0.34 \pm 0.011$
II.....	108	10	$3.01 \pm 0.06$	$8.60 \pm 0.32$	$0.35 \pm 0.014$
III = control	--	10	$2.87 \pm 0.10$	$12.7 \pm 0.43$	$0.23 \pm 0.011$

corresponded to 5 g of fresh liver and contained 0.18 g of solids with an ash content of 9.3 per cent. To the animals of the first experimental group 0.3 ml of this extract was given every day per orally. Each of the second group obtained in the same way 0.6 ml of the extract. The individual animal weight and food consumption were determined daily. The experimental time was 22 days. The results are given in Table 1.

The average daily weight gain was the same in the two experimental groups and in the control group which is in accordance with the previously obtained results (Ågren 1947). The quotient obtained by dividing the daily food consumption of either of the two experimental groups with the corresponding value from the control group is 0.68. The same quotient in the previous investigation was 0.67. The addition of a small amount of a casein digest or a liver extract to the food in both cases diminished the food intake with about 30 per cent. There was no difference in the growth rates between either of the two experimental series with liver extract and the control series. The impression gained by present and previous investigations is that a maximal growth rate is soon attained and is not easily surpassed by the addition of different amounts of growth factors in liver extracts or casein digests to the food of the rat.

It may be pointed out that "sparing effects" have been observed in other nutritional experiments with the rat. There is now common acceptance of the idea, that fat "spares" vitamin B<sub>1</sub> (EVANS and LEPKOWSKY 1928, ARNOLD and ELVEHJEM 1939). Of interest is also the results of RICHTER and BARCLARE 1939 who found that rats fed a synthetic diet without the vitamin B-complex, and allowed to choose their food, ate large amounts of fat but very little of sugar. When fed thiamine, the rats choose sugar preferentially to the fat. In the present investigation the

rats given a small amount of liver extract choosed to reduce their food intake with more than 30 per cent. At the present a satisfactory explanation of this interesting result can not be given.

The mouse bread composed by GARD contains the following ingredients:

Dried bread	500	Meat powder	50
Whole oats	200	Fish liver oil	2—3
Wheat germ	150	Defatted milk	
Alfalfa meal	100	Quant. suff. about	500

The constituents are finely ground, mixed with the amount of milk necessary to make a thick dough, formed to cakes of about 10 g weight, and dried at 38° C. An increase of the B-vitamins by additions of yeast was without visible effect (GARD).

Of special interest is the statement of the baking temperature. This seemed to exclude any possible destruction of the essential amino acids lysine, arginine or thryptophan during the preparation of the bread (PATTON et al. 1948). Analysis showed that the bread contained about 20 per cent of protein. The residue mainly consisted of carbohydrate and fat while the ash content was 4 per cent. The protein intake of the control group was about 2.5 g. If the total amount of nitrogen in the liver extract was considered as protein nitrogen derived from a protein with a nitrogen content of 16 per cent, the animals in group I obtained an addition of about 0.03 g of protein to their preexperimental protein intake of 2.5 g which corresponds to an increase of a little more than 1 per cent. It is not very probable that the amino acids in these 30 mg would considerably change the amino acid pattern of the consumed food protein in a more favourable direction, especially when the bread already contained meat powder. The presence in the liver extract of unidentified factors which facilitates more efficient metabolic conditions seemed a more probable alternative.

### Summary.

The administration of small amounts of a liver extract to young rats significantly increased the weight gain per g food eaten.

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## The Effect of *l*-Noradrenalin and Ergotamine on the Oxygen Consumption of Guinea-Pigs.

By

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In recent years cogent reasons have been adduced in support of the supposition that noradrenalin (Arterenol) is the substance by which the impulses of the sympathetic nervous system are transmitted to effector organs (BACQ 1934, STEHLE and ELLSWORTH 1937, PINKSTON, GREER, BRANNON and BAXTER 1937, 1938, MELVILLE 1937, v. EULER 1946 a, b, c., BACQ and FISCHER 1947, SCHMITERLÖW 1948, HOLTZ and SCHÜMANN 1949).

In some sympathetic nerve-endings, however, adrenalin is presumably discharged; v. EULER (1948) has accordingly divided the sympathetic transmitter into two separate groups, which he terms "sympathin N" and "sympathin A". The former shows the properties of noradrenalin, the latter those of adrenalin. — The pharmacological distinction between noradrenalin and adrenalin has attracted increasing interest.

Summing up the results of the discussion on this subject, it has been found that only on the blood pressure and on the uterus of pregnant cats has noradrenalin a stronger effect than adrenalin and that on other functions the effect of adrenalin is more marked (WEST, 1947).

The ratio between the dosages of noradrenalin and adrenalin, respectively, that have the same effect on a function depends on what optic isomers of the two drugs are compared. On the blood pressure the effect of *d*-noradrenalin is merely 3—4 per cent of that of *l*-noradrenalin (TAINTER, TULLAR and LUDUENA, 1948).

This is clearly indicated by the fact that the ratio between "equipressor" dosages of *dl*-noradrenalin and *dl*-adrenalin is 0.67—0.8: 1 (BARGER and DALE, 1910) and between *l*-noradrenalin and *l*-adrenalin 0.61: 1 (TAINTER *et al.*, *op. cit.*), whereas the said ratio between *dl*-noradrenalin and *l*-adrenalin is 1.2: 1 (TAINTER, 1931). As regards other functions, the ratio between *dl*-noradrenalin and *l*-adrenalin ranges from 0.8: 1 for the uterus of pregnant cats to 100: 1 for the uterus of non-pregnant rats (WEST *op. cit.*).

The effect of noradrenalin on the metabolism has been studied by THIBAUT (1948), who compared the effect of 2 mg/kg noradrenalin (the optic activity not mentioned) with 0.75 mg/kg adrenalin on the oxygen consumption of white rats. The drugs were supplied in the form of subcutaneous injections. These dosages produced an equally marked initial rise of the oxygen consumption (65—85 %), but the effect of noradrenalin was of shorter duration. From this the said author concludes that noradrenalin has a weaker and more evanescent effect than adrenalin. This conclusion, however, is open to the objection that the duration of the more rapid metabolic rate (after subcutaneous injection) depends on how quickly the drug is resorbed, whence, in comparing the effects of adrenalin and noradrenalin, differences in duration are of minor interest.

According to my own investigations on the oxygen consumption of guinea-pigs, a graded response is obtained only between 4 and 100  $\gamma$ /kg of adrenalin on subcutaneous injection. The dosages used by THIBAUT therefore seem to be too large to facilitate quantitative comparisons. With due regard to these factors, the effect of *l*-noradrenalin<sup>1</sup> was compared with that of *l*-adrenalin. The inhibitory effect of ergotamine on the larger oxygen consumption after administration of noradrenalin was likewise investigated. The effect of ergotamine in different concentrations on the oxygen consumption was also noted.

### Procedure.

The oxygen consumption was recorded with an apparatus which has previously been described (LUNDHOLM and MOHME 1949). The investigation was made on guinea-pigs who had been kept without food for 16—20 hours. The temperature of the water-bath was in

<sup>1</sup> From Winthrop Inc. Co., New York; the preparation was kindly placed at my disposal by Professor U. S. v. EULER.

some tests 26° C., in others 32° C. The tests were made on four guinea-pigs simultaneously, and their total gas exchange was determined. The advantage of this procedure is that the variation which may have been regarded as an error is reduced to half. The number of tests that would have to be made in order to show that the differences are statistically significant can therefore be considerably lessened (LUNDHOLM 1949 a). Noradrenalin as well as ergotamine were supplied in 1 cc of 0.9 % NaCl. Noradrenalin was administered in a dorsal subcutaneous injection, ergotamine (Gynergen, Sandoz) in an intramuscular injection into one hind-leg.

The course of the procedure was, generally speaking, as follows: When the animals had been placed in the respiration chamber, the oxygen consumption was first recorded after the lapse of 10 minutes and afterwards in periods of 10 minutes during one hour. The animals were then removed from the respiration chamber and the different drugs were injected. All the injections were made in the course of 2—3 minutes. Ten minutes after the last injection the determination of the oxygen consumption was resumed. — In the tests where ergotamine as well as noradrenalin were administered the ergotamine was first injected and 20 minutes later the noradrenalin; when 10 more minutes had elapsed the oxygen consumption was recorded.

### Results.

All the tests, except those with 2 mg ergotamine per kg, were made on 3 groups of four guinea-pigs, that is altogether 12 animals. Having recorded the basal oxygen consumption in cc per minute for the whole group of 4 animals, the effect of the different drugs is shown in percentage of the basal value. The basal values for the different groups in some cases vary considerably, which is due partly to the fact that some of the tests were made at a temperature of 26°, others at 32°; partly that the tests had been extended for such a length of time that the animals in the meanwhile had increased in weight. The extreme limits for the variations in weight have therefore been indicated as regards each individual animal. As it could not be found that the effects of the drugs had been at all affected by the different temperatures, this matter will not require further discussion.

The results of the tests are recorded in Tables 1 and 2. The statistical analysis will be found in Tables 3 and 4. The time-action curves of the effects of the different drugs on the oxygen consumption are indicated in the Figures. On the ordinate the changes in the oxygen consumption are shown in percentage of the basal value. — Each point represents the average of 6—12 tests.

*Control tests.* The animals were subjected to 6 control tests.



The effects of *l*-noradrenalin and ergotamine tartrate on the oxygen consumption.  
*basal value measured*

Group No.	Range of weight of each of the four animals gm	20 $\gamma$ /kg <i>l</i> -noradrenalin		100 $\gamma$ /kg <i>l</i> -noradrenalin		20 $\gamma$ /kg ergotamine 20 $\gamma$ /kg <i>l</i> -noradrenalin	
		Basal value O <sub>2</sub> cc/min.	Per cent of basal value 10—40 min. after injection of the drug	Basal value O <sub>2</sub> cc/min.	Per cent of basal value 10—70 min. after injection of the drug	Basal value O <sub>2</sub> cc/min.	Per cent basal va 10—4 min. af injection of nor adrena
I ♂	1. 100—1 200	33.9	110.1	33.9	117.5	31.5	111.4
	2. 730— 860	33.1	102.2	34.2	122.6	30.7	109.3
	3. 930—1 000	31.2	106.2	34.2	124.8		
	4. 830— 910						
II ♀	1. 820— 910	27.8	112.5	31.3	124.3	25.9	110.6
	2. 810— 970	27.3	106.8	32.6	103.0	25.6	109.1
	3. 700— 850	27.0	108.8	31.6	108.4		
	4. 750— 840						
III ♀	1. 550— 920	29.0	126.0	30.7	109.0	26.3	123.5
	2. 660—1 070	27.2	116.5	31.2	107.5	25.6	108.9
	3. 500— 760	26.8	111.6	31.4	105.8		
	4. 560—1 000						

They first received a subcutaneous injection of 1 cc 0.9 % NaCl, whereupon the oxygen consumption was recorded for 60 minutes. Afterwards 1 cc of the said saline solution was injected intramuscularly into the one hind-leg, and the oxygen consumption was measured for 90 minutes. It is evident from Fig. 1 that no effect on the oxygen consumption can be discerned for 10—20 minutes after the injections, which corresponds with previous results (LUNDHOLM and MOHME *op. cit.*). Tables 1 and 3 indicate also that the oxygen consumption was quite constant during the 150

1.

oxygen consumption after the injections of the drugs is given in percentage of the min. before the injections.

200 $\gamma$ /kg ergotamine 20 $\gamma$ /kg l-noradrenalin		20 $\gamma$ /kg ergotamine		200 $\gamma$ /kg ergotamine		Control tests	
Basal value O <sub>2</sub> cc/min.	Per cent of basal value 10—40 min. after injection of nor- adrenalin	Basal value O <sub>2</sub> cc/min.	Per cent of basal value 10—100 min. after injection of the drug	Basal value O <sub>2</sub> cc/min.	Per cent of basal value 10—70 min. after injection of the drug	Basal value 10—70 min. after injection (subcutane- ously) of 1 cc 0.9 % NaCl O <sub>2</sub> cc/min.	Per cent of basal value 10—70 min. after injection (intramus- cularly) of 1 cc 0.9 % NaCl.
30.4	96.5	33.4	114.6	30.5	99.0	31.7	101.3
33.2	99.7	34.5	112.1	30.0	101.8	31.2	100.0
35.0	112.8	32.9	105.7	35.7	102.8		
		33.3	93.8	31.1	99.6		
27.7	107.0	31.1	117.2	25.8	98.9	33.4	100.5
28.0	99.5	30.3	110.1	27.1	93.9	31.5	98.3
32.9	94.7	33.2	92.9	31.3	96.4		
		32.0	100.3	34.0	89.2		
24.9	104.7	32.1	102.4	24.2	90.9	34.2	99.8
25.2	97.7	29.9	100.8	22.7	98.8	32.2	100.0
32.6	92.5	32.6	104.8	31.8	93.0		
		31.8	107.8	38.0	92.7		

minutes for which the test proceeded. The standard deviation for the 15 periods of 10 minutes was 1.8 %. Thus, according to the t-test, the probability that a particular mean value for a period of 10 minutes, based on 6 observations, owing to chance will exceed  $\pm 4$  % is 0.05. This may serve for guidance in judging the figures. Table 3 indicates that the standard deviation for the control tests 10—70 minutes after the intramuscular injection is merely 1 %. The probability that in a particular test greater deviations than  $\pm 4$  % may be due to chance is 0.01.

Table 2.

*The effect of 2 mg/kg of ergotamine tartrate on the oxygen consumption.*

Group No.	Weight of each animal gm.	2 mg/kg ergotamine		
		Basal value O <sub>2</sub> cc/min.	Per cent of basal value 10—40 min. after injection of the drug	Per cent of basal value 40—190 min. after injection of the drug
V.	940	31.1	104.7	130.5
	825			
	760	30.3	109.9	129.0
	730			
VI.	680	25.6	96.8	110.2
	610			
	580	25.2	97.2	115.1
	460			
VII.	730	27.8	88.5	103.8
	640			
	630	28.4	97.6	118.3
	570			
VIII.	560	25.1	88.7	132.8
	550			
	510	22.6	98.9	122.6
	480			

*Noradrenalin tests.* The effects on the oxygen consumption (1) of 20  $\gamma$ /kg and (2) of 100  $\gamma$ /kg of *l*-noradrenalin were investigated severally in 9 tests. From Fig. 1A it may be seen that the oxygen consumption ensuing from the supply of 20  $\gamma$ /kg rose at its highest to 116 % and that the effect had completely ceased 40 minutes after the injection. When the supply was increased to 100  $\gamma$ /kg the oxygen consumption rose to 125 % at its highest, but 70 minutes after the injection had fallen to 108 %. If we take the *mean* effect of the drug, it will be found that in consequence of supplying 20  $\gamma$ /kg the oxygen consumption showed an average rise of 11.2 % in the course of 10—40 minutes after the injection; and that when the supply was increased to 100  $\gamma$ /kg the oxygen consumption rose on an average by 13.7 % within 10—70 minutes from the injection. In both cases the rise was statistically significant. — For purpose of comparison, the effect of 4  $\gamma$ /kg, 20  $\gamma$ /kg and 100  $\gamma$ /kg of *l*-adrenalin is shown in Fig. 1B.

These tests were made with precisely the same technique as those with noradrenalin. A detailed account of the latter is given

Table 3.

*Statistical analysis of the values in Tables 1 and 2.*

Variate	Increase or decrease percent. Mean.	Number of test.	Standard error of the mean	t.	P.
Control tests 10—70 min. after the intramuscular injection	0.0	6	$\pm 0.403$		
20 $\gamma$ /kg l-noradrenalin 10—40 min. after the injection .....	+ 11.2	9	$\pm 2.302$	4.861	0.001—0.01
10—70 min. after the injection .....	+ 6.4	9	$\pm 1.643$	3.877	0.001—0.01
100 $\gamma$ /kg l-noradrenalin 10—70 min. after the injection .....	+ 13.7	9	$\pm 2.874$	4.753	0.001—0.01
4 $\gamma$ /kg l-adrenalin 10—70 min. after the injection .....	+ 5.3	9	$\pm 0.946$	5.652	<0.001
20 $\gamma$ /kg l-adrenalin 10—70 min. after the injection .....	+ 14.8	14	$\pm 2.161$	6.849	<0.001
100 $\gamma$ /kg l-adrenalin 10—70 min. after the injection .....	+ 24.3	30	$\pm 1.800$	13.500	<0.001
20 $\gamma$ /kg ergotamine 10—100 min. after the injection .....	+ 5.2	12	$\pm 2.209$	2.354	0.02—0.05
30—60 min. after the injection .....	+ 5.0	12	$\pm 1.985$		
200 $\gamma$ /kg ergotamine 10—70 min. after the injection .....	— 3.6	12	$\pm 1.269$	2.837	0.01—0.02
30—60 min. after the injection .....	— 2.9	12	$\pm 1.712$		
2 mg/kg ergotamine 10—40 min. after the injection .....	— 2.2	8	$\pm 2.557$	0.900	0.3—0.4
40—190 min. after the injection .....	+ 20.3	8	$\pm 3.651$	5.557	<0.001
20 $\gamma$ /kg ergotamine and 20 $\gamma$ /kg l-noradrenalin 10—40 min. after the injection of noradrenalin .....	+ 12.1	6	$\pm 2.309$	4.855	0.001—0.01
200 $\gamma$ /kg ergotamine and 20 $\gamma$ /kg l-noradrenalin 10—40 min. after the injection of noradrenalin .....	+ 0.6	9	$\pm 2.211$	0.271	0.7—0.8

Table 4.

*Statistical analysis of the values in Tables 1 and 2.*

Difference between:	Difference	Degrees of freedom	Standard error of the difference	t.	P.
Sum of 20 $\gamma$ /kg <i>l</i> -noradrenalin and 20 $\gamma$ /kg ergotamine reduced with 20 $\gamma$ /kg ergotamine 20 $\gamma$ /kg noradrenalin ( $A+B-AB$ ) .....	+ 4.1	27	$\pm 3.816$	1.074	0.2—0.3
Sum of 20 $\gamma$ /kg <i>l</i> -noradrenalin and 200 $\gamma$ /kg ergotamine decreased with 200 $\gamma$ /kg ergotamine 20 $\gamma$ /kg noradrenalin. ( $A+B-AB$ ) ...	+ 7.7	27	$\pm 3.621$	2.127	0.02—0.05
20 $\gamma$ /kg <i>l</i> -adrenalin and 20 $\gamma$ /kg <i>l</i> -noradrenalin	+ 8.4	23	$\pm 2.715$	3.094	0.001—0.01
100 $\gamma$ /kg <i>l</i> -adrenalin and 100 $\gamma$ /kg <i>l</i> -noradrenalin	+ 10.6	37	$\pm 3.391$	3.126	0.001—0.01

elsewhere (LUNDHOLM 1949 b). As shown by Tables 3 and 4, the effects ensuing from the supply of 20 and 100  $\gamma$ /kg adrenalin have been found with statistical significance to be greater than those resulting from the supply of corresponding doses of noradrenalin. It may be seen from Table 3 and Fig. 1 that the time-action curves for 4  $\gamma$ /kg adrenalin and 20  $\gamma$ /kg noradrenalin are similar both as regards the maximum increase and its duration. The effect of the noradrenalin in these respects, however, is somewhat stronger. A similar remark applies also to the curves for 20  $\gamma$ /kg adrenalin and 100  $\gamma$ /kg noradrenalin, though in this case the effect of the adrenalin is stronger. It thus appears that the ratio between the dosages of noradrenalin and adrenalin with the same effect on the oxygen consumption is about 5 : 1.

*Ergotamine-noradrenalin tests.* In order to prove that a drug inhibits the effect of another drug on a function, it must be shown that  $A + B - AB > 0$  (where A is the effect of the blocked substance, B the effect of the blocking substance, and AB the simultaneous effect of the two substances on the function). It is rather often assumed by investigators that  $B = 0$  and it is considered sufficient to show that  $A - AB > 0$ . The

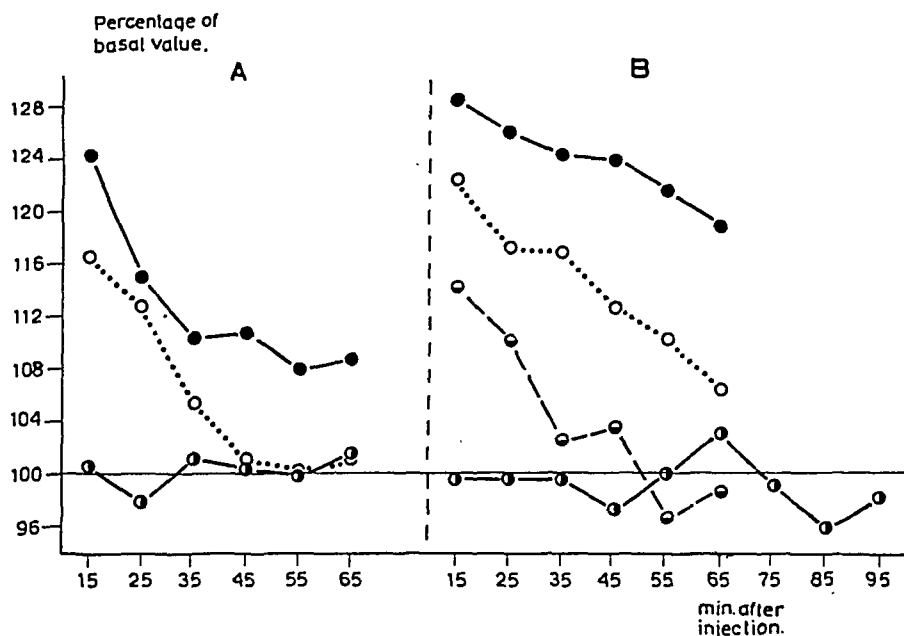


Fig. 1. Time-action curves for *l*-noradrenalin and *l*-adrenalin.

Abscissa: time after injection of drug. Ordinate: Oxygen consumption in percentage of basal value, measured for 60 minutes before injection.

A. *l*-noradrenalin: 20  $\gamma$ /kg  $\circ \cdots \circ$ . 100  $\gamma$ /kg  $\bullet \cdots \bullet$ . B. *l*-adrenalin: 4  $\gamma$ /kg  $\bullet \cdots \bullet$ . 20  $\gamma$ /kg  $\circ \cdots \circ$ . 100  $\gamma$ /kg  $\bullet \cdots \bullet$ .

Control test  $\circ \cdots \circ$ . A. after subcutaneous injection. B. after intramuscular injection.

risk of confusing inhibition with antagonism in that case is evident.

It has previously been shown (LUNDHOLM and MOHME *op. cit.*) that 20  $\gamma$ /kg ergotamine completely inhibited the effect of 20  $\gamma$ /kg adrenalin on the oxygen consumption. We therefore investigated whether 20  $\gamma$  ergotamine inhibited the effect of 20  $\gamma$ /kg noradrenalin. As we see from Figs. 1 A and 2 A, the curves for 20  $\gamma$ /kg noradrenalin and 20  $\gamma$ /kg ergotamine plus 20  $\gamma$ /kg noradrenalin almost coincide, and there does not appear to be any inhibition. Table 4 shows, however, that the difference between 20  $\gamma$ /kg noradrenalin + 20  $\gamma$ /kg ergotamine and 20  $\gamma$ /kg ergotamine and 20  $\gamma$ /kg noradrenalin simultaneously (A + B - AB) is 4.4 %. This difference has not indeed been statistically verified, but, as it deviates from 0, a certain degree of inhibition cannot be ruled out. As may be seen from Table 4, the corresponding difference in tests with 20  $\gamma$ /kg noradrenalin and 200  $\gamma$ /kg ergotamine is 7.7 % and statistically probable. From Fig. 2 it may also be seen that the curves for 200  $\gamma$ /kg ergotamine and for 200  $\gamma$ /kg ergotamine plus 20  $\gamma$ /kg noradrenalin are almost

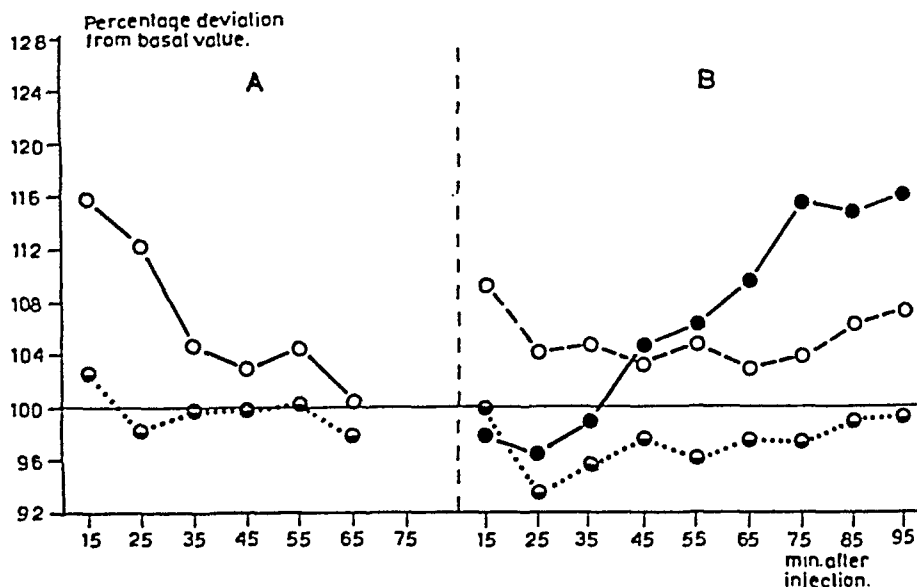


Fig. 2. Time-action curves for ergotamine plus *l*-noradrenalin (A) and ergotamine (B).

A. 20  $\gamma$ /kg ergotamine + 20  $\gamma$ /kg *l*-noradrenalin,  $\bigcirc$ — $\bigcirc$ ; 200  $\gamma$ /kg ergotamine + 20  $\gamma$ /kg *l*-noradrenalin  $\bigcirc$ ..... $\bigcirc$ .

B. Ergotamine. 20  $\gamma$ /kg  $\bigcirc$ — $\bigcirc$ . 200  $\gamma$ /kg  $\bigcirc$ ..... $\bigcirc$ . 2 mg/kg  $\bullet$ — $\bullet$ .

identical, indicating that the effect of the noradrenalin is blocked. — It should be pointed out that in these estimates the effect of the ergotamine on the oxygen consumption was measured 30–60 minutes after the injection, so that the lapse of time from the ergotamine injection should be the same both in the tests with ergotamine solely and in those with noradrenalin plus ergotamine. — The individual values for these periods are not indicated, but merely the mean values, which will be found in Table 3.

*Ergotamine tests.* The effect of different doses of ergotamine on the oxygen consumption proved to be rather complicated. After the supply of 20  $\gamma$ /kg the oxygen consumption rose on an average by 5.2 % and statistical analysis indicated that this figure was probably correct; but towards both high and low values the variation in the separate tests was considerable. As may be seen from the control tests it is merely in one case out of a hundred that a value lies beyond the limits 96 %–104 %. Table 1 shows, however, that the values after the supply of 20  $\gamma$ /kg lie both above and below the said figures. It may accordingly be concluded that the effect of this dosage on the metabolism is in some cases stimulative, in others inhibitory. This conclusion is borne out by

the tests with 200  $\gamma$ /kg where the oxygen consumption, with statistical probability, showed a fall of 3.6 %. The effect of 2 mg/kg is two-phase (Fig. 2 B, Table 2). The oxygen consumption falls at first to 96 per cent of the basal value at the lowest, but then gradually rises to about 120 per cent 90—180 minutes after the injection (not included in Fig. 2). Though the initial fall of the oxygen consumption has not been statistically verified, it is probable in the light of the preceding tests with 200  $\gamma$ /kg that it actually occurred, and may be explained on the supposition that with increasing absorption the effect of ergotamine on the oxygen consumption is modified. It seems thus as if small doses of ergotamine tended to stimulate the metabolism, that somewhat larger doses had an inhibitory effect thereon, whereas very large doses were again stimulative.

### Discussion.

The ratio 5 : 1 between doses of *l*-noradrenalin and *l*-adrenalin with the same effect on the metabolism corresponds with the fact that the effect of adrenalin in many cases is stronger than that of noradrenalin. It also bears out Thibault's statement that noradrenalin has a weaker effect on the metabolism than adrenalin.

It has previously been shown that the effect of noradrenalin on the blood pressure is wholly or partially inhibited by adreno-lytic drugs such as ergotoxine (BARGER and DALE 1910), ergotamine (STEHLE and ELLSWORTH 1937), F 933 and F 883 (MELVILLE 1937) and dibenamin (FOLKOW, FROST and UVNÄS 1948). Exact quantitative determinations of the ratio between the doses of the said drugs required in order to inhibit the effects of adrenalin or noradrenalin, respectively, are apparently not available. The above-reported tests, however, indicate that as regards noradrenalin a larger dose of ergotamine is required for the purpose. It cannot, however, be inferred from these tests that *e. g.* a ten times larger dose would be requisite, as the doses which cause an equally marked (say 50 %) inhibition of the effect of noradrenalin and adrenalin have not been compared. This may perhaps account for the fact that the attempts made completely to abolish the "pressor" effect of noradrenalin with doses of ergotoxine (BARGER and DALE *op. cit.*) or ergotamine (EULER 1946 c) which conduce to reverse the effect of adrenalin have not always been successful.



The difficulty of completely abolishing the rise of the blood pressure after stimulation of the hepatic nerves with ergotoxine (CANNON and ROSENBLUETH 1937, GADDUM and GOODWIN 1946) may likewise be explained in this way, as also the finding of HOUSAY and GERSCHMAN (1947) that doses of ergotamine or dihydroergotamine which inhibited the glycogenolytic effect of adrenalin had no such inhibitory effect on noradrenalin.

The effect of ergotamine on the metabolism seems to be rather complicated: three different factors must apparently be taken into account. According to AHLGREN (1924) and v. EULER (1929), low concentrations of ergotamine ( $10^{-11}$ ) stimulated the metabolism of isolated tissues. It may therefore be presumed that the effect of 20  $\gamma$ /kg of ergotamine is due to a direct stimulation of the metabolism of the tissues.

The inhibitory effect of ergotamine has been observed by a number of investigators (ABDERHALDEN and WERTHEIMER 1927, MARINE, DEUTSCH and CIPRA 1927, MICHAEL, BENDESCU and VANCEA 1928, HARANGOZO-OROŚZY and ISSEKUTZ 1942, LUNDHOLM and MOHME *op. cit.*). According to MOHME (1949) this effect appears to be due to a blockade of the normal sympathetic impulses. The stimulating action of very large doses of ergotamine is presumably a central effect (ROTHLIN 1923). The animals were markedly agitated after administration of 2 mg/kg, so that the greater consumption of oxygen seems, at any rate in part, to be due to motor agitation. — A more rapid metabolic rate after the supply of large doses of ergometrine has previously been observed in white rats by DAVIS, ADAIR, CHEN and SWANSON (1935).

Ergotamine does not lower the basal oxygen consumption of guinea-pigs in a dosage that inhibits the effect of adrenalin on the metabolism. From this it may be concluded that adrenalin endogenously secreted has no effect at all on the basal metabolism of this animal. On the other hand, a dosage of ergotamine that inhibits the effect of noradrenalin tends to lower the basal oxygen consumption. This corresponds firstly with the theory that noradrenalin is the substance which transmits the impulses from the sympathetic nervous system, and secondly with the supposition that the lowered metabolic rate is due to an inhibition of the normal sympathetic impulses.

### Summary.

The effects of *l*-noradrenalin and ergotamine on the oxygen consumption have been studied in guinea-pigs. The drugs had been supplied in the form of subcutaneous and intramuscular injections, respectively.

It was found that 20  $\gamma$ /kg *l*-noradrenalin raised the oxygen consumption to at most 116 per cent. of the basal value, but that the effect had ceased after 40 minutes; that 100  $\gamma$ /kg caused a maximal rise of the consumption to 125 per cent., and that the effect still persisted after the lapse of 70 minutes.

The ratio between the dosages of *l*-noradrenalin and *l*-adrenalin which have an equally marked effect on the oxygen consumption has been estimated at 5 : 1.

It was found that ergotamine inhibited the effect of *l*-noradrenalin on the oxygen consumption; but that a considerably larger dosage of ergotamine was requisite in order to inhibit the effect of *l*-noradrenalin than the corresponding dosage required in the case of *l*-adrenalin.

The effect of ergotamine on the metabolism was found to vary with the dosage. Thus, 20  $\gamma$ /kg had in the main a slightly stimulating effect: the oxygen consumption rose on an average to 105.2 per cent. of the basal value 10—100 minutes after the injection; 200  $\gamma$ /kg lowered the oxygen consumption to 96.4 % within the same time; 2 mg/kg at first lowered the oxygen consumption, but this was afterwards followed by a stimulative effect, the result being that the oxygen consumption rose to 120.3 % in the course of 40—190 minutes after the injection.

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## The Variation in Activity of Apodehydrogenases During Insect Metamorphosis.

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In a previous paper (AGRELL 1947) it was established that the integral oxidative metabolism in vivo and the spontaneous dehydrogenase activity in vitro show closely the same variation during the metamorphosis of the fly *Calliphora erythrocephala* MEIG. Similar U-shaped curves are found in both cases, and this fact has been still more substantiated by further series of tests. Therefore it can be stated, that the minimum in oxidative metabolism principally depends upon a corresponding variation in activity of the hydrogen activating enzyme systems, *i. e.* of the dehydrogenase systems. However, it was not clear if this variation is caused in the first place by a change in spontaneous donor or coenzyme concentration or by a change in activity of the apoenzymes. The present paper will try to deal with this problem.

### Material and Methods.

The material on which this investigation is based comprises, as before, pupae in all developmental stages of the fly *Calliphora erythrocephala* MEIG. The breeding of larvae and pupae took place at a temperature of  $+ 22^{\circ}\text{C}$ .

The measurements of the enzyme activity were made with the methylene blue technique of THUNBERG at  $+ 25^{\circ}\text{C}$ . For determination of the activity of the apodehydrogenases three pupae were thoroughly crushed, washed with 10 ml distilled water and centrifuged (5,000 R/M). The procedure was repeated three times. More washings gave no further decrease in spontaneous activity. The sediment was transferred to

Thunberg-tubes containing 0.25 ml methylene blue (Mb) solution in a concentration of 1 : 25,000, 0.45 ml Na-K-phosphate buffer of M/15 and pH 7.2 and 0.30 ml of the respective donor substances of M/10. In the cases of the dehydrogenases requiring a coenzyme, 0.1 ml of the buffer solution was replaced by a codehydrogenase I preparation of approx. 30 % purity and in a concentration of 1 ‰, prepared according to LEPAGE 1947. The preparation may also contain codehydrogenase II, because it acts upon the citric dehydrogenase (iso-citric dehydrogenase + aconitase), or, which is also not excluded, the enzyme in question may cooperate with codehydrogenase I. As donor substances the following were used: the potassium salts of l-malic, succinic, citric, l-glutamic, palmitinic and  $\beta$ -hydroxybutyric acid, the lithium salt of lactic acid and l-alanine, d-glucose, propanol-1, butanal as well, all substances in highly purified form.

The technique by which the vitreous mass is freed from coenzyme and donor substances by washing and centrifugation is not ideal, especially because it may be supposed that a lesser or greater part of the apoenzymes is simultaneously washed away. This may be the case for instance concerning the fatty acid and aldehyde dehydrogenases, as an addition of the corresponding donors to the unwashed pupal mass brings about a high increase in Mb-reduction. Therefore the author has also tried the dialyse method, which, however, does not work. During the dialyse the pupal mass darkens to a very large extent, caused by an oxidative melanine formation, which prevents a later use of the Mb-method. Moreover, even if the dialyse is carried out anaerobically, up to 24 hours at +2° C, the decrease in spontaneous activity is too small, possibly depending upon the non-diffusion of glycogen. For most of the investigated apoenzymes on the other hand the washing-centrifuging technique seems applicable. Cautiousness must, however, be observed in comparing the activity of the different enzymes.

The results are recorded as curves in Fig. 1. Each point represents the mean value of 5—10 single tube tests. The enzyme activity is evaluated as the rate of Mb-reduction,  $100 \times$  the inverse value of decolorization time in minutes. For comparison the curve for the spontaneous Mb-reduction is given in Fig. 2, as a combination of some ten different test series. Also this curve refers to the values established for three pupae.

## Results.

The results are apparent from Fig. 1. As can be seen, some of the apoenzymes which are probably the most important and quantitatively the most prominent, show an obvious U-shaped variation in the Mb-reductive power, *i. e.*, in activity, during the metamorphosis. Thus it can be stated, that the minimum in oxidative metabolism occurring during the metamorphosis of the fly in question, and probably of all holometabolic insects, chiefly

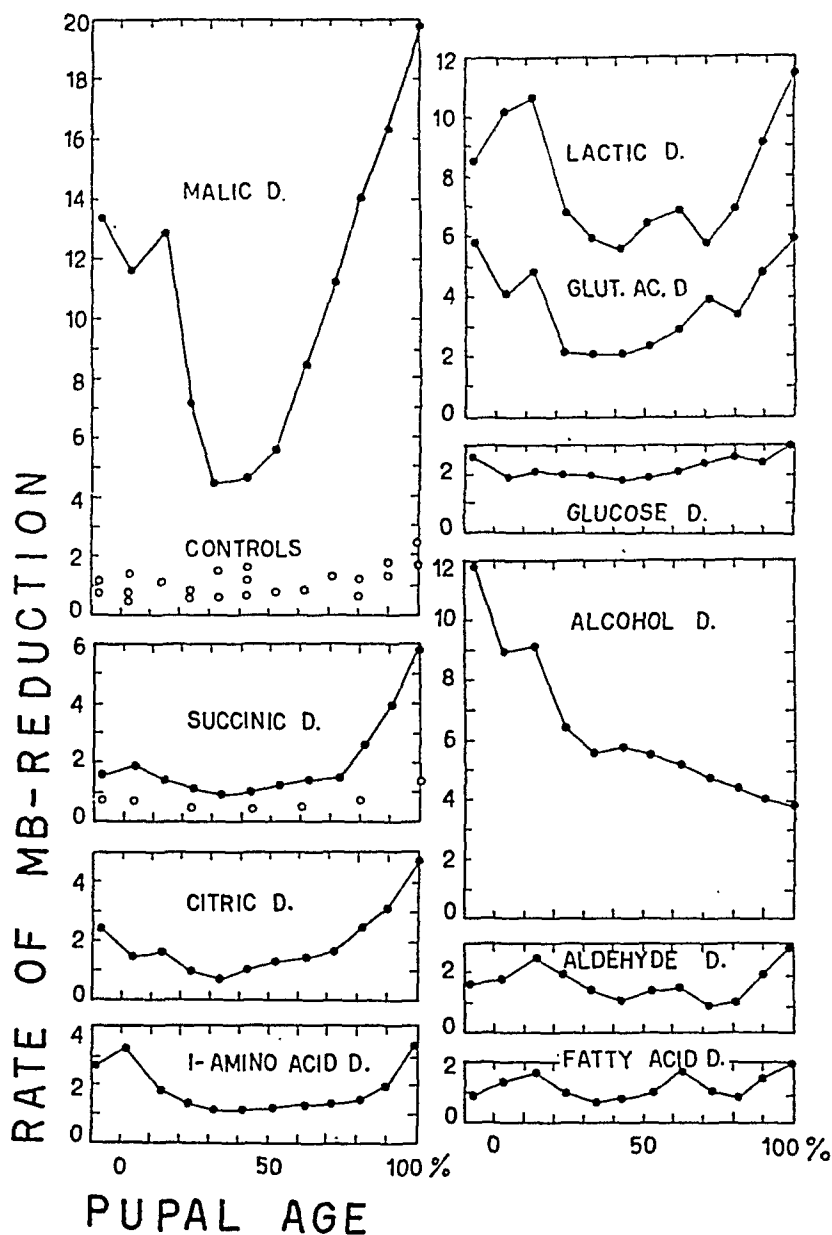


Fig. 1. The activity of some apodehydrogenases during the metamorphosis of the fly *Calliphora erythrocephala* MEIG. The controls below the malic dehydrogenase curve represent an addition to the vitreous mass of coenzyme only, without donors. The controls below the succinic dehydrogenase curve represent an addition of neither coenzyme nor donors.

depends upon a reduced activity of apodehydrogenases and perhaps of diaphoreses. Only an inconsiderable relative deficit in the native concentration of codehydrogenase I could be detected, at the middle of the pupal period, Fig. 3. As a close relation

has been established between apodehydrogenase activity and histolysis-histogenesis processes (AGRELL 1949 a and b) it seems reasonable to suppose a decomposition followed by a reconstruction of this protein part of the enzyme systems.

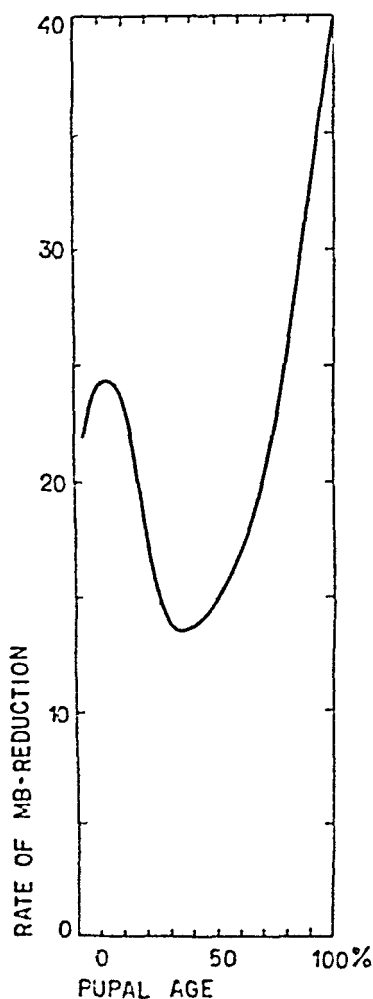


Fig. 2. The spontaneous dehydrogenase activity during metamorphosis.

To judge from its high activity and close agreement in variation with the total dehydrogenase activity, Fig. 1 and 2, the malic dehydrogenase can be considered to hold a central position in the oxidative metabolism of the investigated insect. The small extra minimum in the activity of this enzyme at 0 % pupal age, at the formation of the puparium, is significant, as it appears in all test series of this kind. Strangely enough this minimum is not found if the activity of the enzyme is determined in different parts of the pupa, head, thorax and abdomen (AGRELL 1949 b). Maybe it is caused by some "synergistic" principle formed from different parts of the body. The same minimum occurs in the variation of the citric and in some test series also of the succinic and the glutamic acid dehydrogenases' activity.

The succinic, malic and citric dehydrogenases should represent the best defined oxidative enzymes in the "tricarboxylic acid cycle". Now the activity of the malic dehydrogenase is registered much higher in the investigated animal than that of the succinic and citric dehydrogenases, even if all three enzymes conform in the variation of their activity, for which latter reason it would be plausible to assume that they belong to the same cycle, Fig. 1. It should be noted, that the activity of the malic dehydrogenase can not have been determined as too high compared with, in any case, the succinic dehydrogenase, which latter enzyme is the more strongly cellbound, and can scarcely be washed away. The comparatively high activity of the malic

dehydrogenase, however, suggests that this enzyme does not only work as an intermediate link in a cycle, but also has a function in the main hydrogen transport. Such a combination of the cycles of KREBS and SZENT GYÖRGYI is made by KREBS 1943. Thus there seems to be no objection to accepting the possibility that the "tricarboxylic acid cycle" does occur in the abovementioned sense in the insect in question.

With reference to the other apodehydrogenases, their variation in activity is evident from Fig. 1, and there is not much to add. The lactic dehydrogenase shows a small maximum on the otherwise U-shaped slope of the curve at the middle of the pupal age. This maximum is significant as it is found in all test series. It is also apparent when investigating different parts of the pupa (AGRELL 1949 b). The activity of the alcohol dehydrogenase is surprisingly high. An addition of different alcohols causes a marked increase in the spontaneous Mb-reduction towards the end of the pupal age (AGRELL 1947). This may indicate a successively reduced concentration of spontaneous donor during the later part of the metamorphosis period.<sup>1</sup> The  $\beta$ -hydroxybutyric dehydrogenase does not seem to function. An addition of the corresponding donor has no effect either on the spontaneous Mb-reduction or on the activity of the washed vitreous mass. The activity of the aldehyde and fatty acid dehydrogenases is presumably registered much too low, as mentioned in the methodological part of this paper.

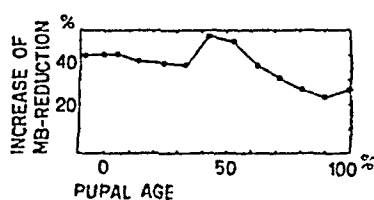


Fig. 3. The relative deficit in the native concentration of codehydrogenase I. The change in rate of the spontaneous Mb-reduction after saturation with this coenzyme.

Valuable technical work in this investigation has been carried out by Miss ELSA ROSENGREN. The investigation has been facilitated by grants from the Scandinavian Insulin Foundation and from the Swedish Research Council for Natural Science.

### Summary.

The activity of some apodehydrogenases has been determined during the entire metamorphosis period of the fly *Calliphora erythrocephala* MEIG. by use of the methylene blue technique.



The minimum in oxidative metabolism occurring during the metamorphosis is traced back to a similarly changed activity of the apodehydrogenases.

The malic dehydrogenase occupies a central position in the oxidative metabolism.

Reasons are put forward for the acceptance of a "tricarboxylic acid cycle" operating in the investigated insect.

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## Inactivation of Thrombin by the Blood of Different Mammals.

By

MIKLÓS D. F. UDVARDY.

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The excess of thrombin formed during the coagulation of the blood is rapidly removed from the plasma, a process believed to be due to metathrombin formation. This process has been thoroughly studied earlier (WÖHLISCH 1940, p. 334).

The disappearance of the thrombin has been ascribed to an adsorption to the blood proteins (RETTGER 1909), to an enzymatic destruction of the thrombin or to both processes (GERENDÁS 1946, 1948).

This mechanism deserves to be studied further, particularly against the background of our present knowledge. In a preliminary paper I attempted to determine the speed of inactivation of thrombin caused by sera of different mammals.

### Experimental.

The inactivation of thrombin was determined as follows. Thrombin of known potency, free of antithrombin, was incubated with serum (plasma or whole blood) and the decrease of thrombin activity tested on fibrinogen. (GERENDÁS 1948.)

The manner of blood sampling varied in different animals. In the case of the horse, cattle, sheep, goat and pig the blood was collected from the severed carotid artery in slaughtering the animals. In sheep, some samples were taken through puncture of the jugular vein. In rabbits and dogs, the blood was removed from the veins of the ear. In mice, I took samples from the carotid artery. In the case of rats and guinea-pigs, as well as the hedgehog I punctured the heart, in some cases under mild ether anesthesia.

Table I.

*Thrombin inactivation by pig, rabbit and cattle serum. Duration of experiment 4 hours. Temperature 24° C.*

Time of testing.	0''	17''	1'	1'40''	2'45''	4'	6'	10'	30'	116'	180'	225'	
Coagul. time	11''	14''	45''	80''	2.5'	4.5'	6.5'	14'	22'	36'	32'	36'	
Time of testing	0''	1'	2'	3'	5'	7'	10'	14'	21'	30'	65'	120'	180'
Coagul. time	11''	17''	22''	27''	43''	64''	122''	4.55'	5.5'	8.5'	13'	17'	16'
Time of testing	0''	1'	2'	3'	5'	7'	9'	12'	20'	35'	62'	96'	180'
Coagul. time	11''	18''	21''	25''	37''	48''	73''	90''	3.5'	4.5'	8.5'	8.5'	8.5'

The experiments were carried out at room temperature (24—26° C). After 20 minutes the blood samples were centrifuged for 10 minutes at 3 500 r. p. m. The experiments were performed within one hour, in the majority of cases within 30—40 minutes.

Roche Thrombin (Hoffman-La Roche, Bâle) was used in all experiments. It was dissolved in physiological saline solution. Fibrinogen was prepared from oxalated ox blood by LAKI's (1942) method, with some modifications. The absorption with tricalcium phosphate was repeated twice. After absorption, the plasma was used as fibrinogen solution. It was poor in prothrombin and could be kept at +4° C for 6 weeks without coagulation or denaturation, even at room temperature for several days. The fibrinogen and the thrombin solutions were diluted before use to give a coagulation time of about 12—15 sec. The coagulation time was determined on a watch glass by means of a glass hook.

### Experiments.

1. Sera of cattle, rabbit and pig were incubated for 4 hours with thrombin as follows:

Serum .....	1.5 ml
Thrombin solution 1 mg/ml .....	1.5 ml

The drop in thrombin activity during incubation was measured by determining the coagulation time in a mixture of:

Fibrinogen solution .....	0.1 ml
Dist. water .....	0.1 ml
Mixture of serum and thrombin .....	0.1 ml

The details of this experiment are summarized in Table I.

In a second series of experiments I investigated the thrombin inactivation of sera of different mammals during a 10 min. incubation time. The average reaction velocity constant for each is found in Table II. In the animals represented in Fig. 1, the

Table II.

*The reaction-velocity coefficients/ $k = 1/t. \log. \text{nat. } C_0/C$  of the thrombin inactivation.*

Species	Nr.	k	Species	Nr.	k
Horse .....	1.	0.255	Goat .....	1.	0.480
	2.	0.280		2.	0.410
	3.	0.265		Average	0.445
	4.	0.280	Rat .....	1.	0.550
	Average	0.270		2.	0.580
Cattle .....	1.	0.185		3.	0.500
	2.	0.270		4.	0.450
	3.	0.365		5.	0.480
	4.	0.270		6.	0.340
	5.	0.270		Average	0.490
	6.	0.245	Mouse.....	1.	0.630
	7.	0.365		2.	0.720
	8.	0.210		3.	0.720
	9.	0.275		4.	0.640
	10.	0.300		5.	0.590
	11.	0.275		6.	0.570
	12.	0.260		7.	0.680
	13.	0.320		8.	0.620
	14.	0.360		9.	0.580
	15.	0.330		10.	0.590
	16.	0.310		11.	0.730
	Average	0.290		12.	0.740
Sheep .....	1.	0.680	Dog .....	Average	0.650
	2.	0.430		1.	0.680
	3.	0.270		2.	0.710
	4.	0.430		3.	0.740
	5.	0.300		4.	0.570
	6.	0.290		Average	0.670
Rabbit .....	Average	0.370	Pig .....	1.	0.700
	1.	0.360		2.	0.630
	2.	0.470		3.	0.630
	3.	0.320		4.	0.770
	4.	0.480		5.	0.850
	5.	0.480		6.	0.670
	6.	0.310		7.	0.820
	7.	0.380	Guinea pig .....	Average	0.720
	8.	0.445		1.	0.780
	9.	0.375		2.	0.700
	10.	0.440		3.	0.830
	11.	0.300		4.	0.890
	12.	0.400		5.	0.840
	13.	0.380		6.	0.820
	14.	0.370		7.	0.780
	15.	0.400		8.	0.770
	16.	0.440		Average	0.800
	Average	0.400	Hedgehog .....	1.	0.830

velocity of the process is constant during the 10 minutes investigated. The reaction velocity constant ( $k = 1/t. \log. \text{nat. } cO/c$ ) was calculated according to GERENDÁS.

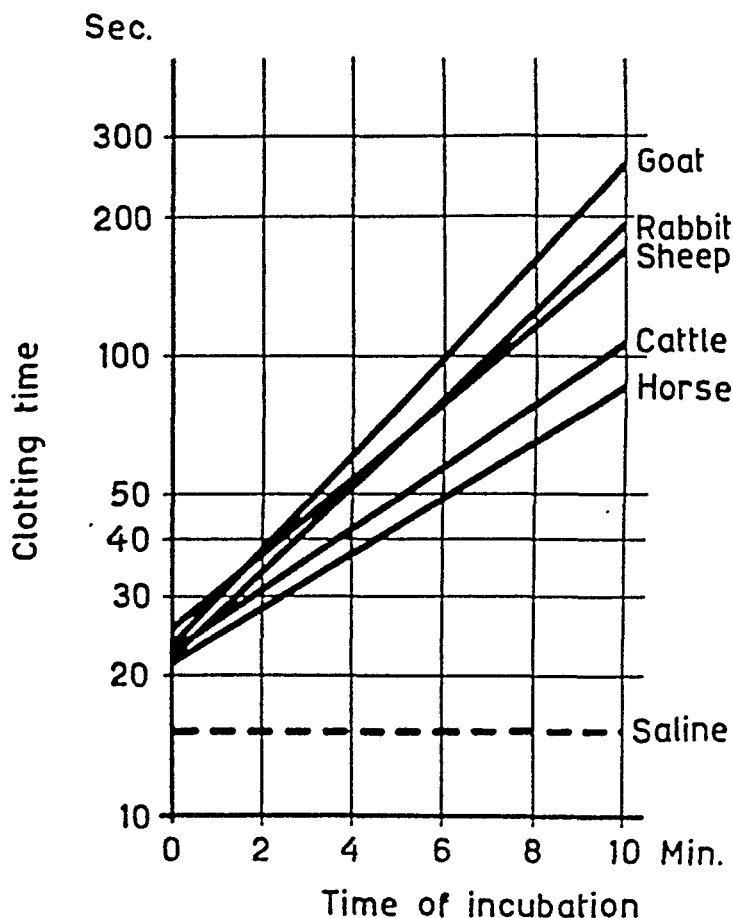


Fig. 1. Thrombin inactivating capacity in sera of group 1 of the species.

In the case of the species represented in Fig. 2, the velocity of the thrombin inactivation process is not constant even with 10 minutes' incubation time. The initial rapid inactivation diminishes continuously. Here, I calculated the average velocity, *i. e.*, the  $k$  value calculated from the initial activity of thrombin, without incubation, and those in the 10th minute of inactivation.

### Discussion.

The most important phase of the inactivation of thrombin lies between the beginning and the 10—12th minute of the inactivation. The sera of the species investigated can be classified from this viewpoint into two markedly different groups, represented by the rabbit, and the pig serum (*v.* Table I).

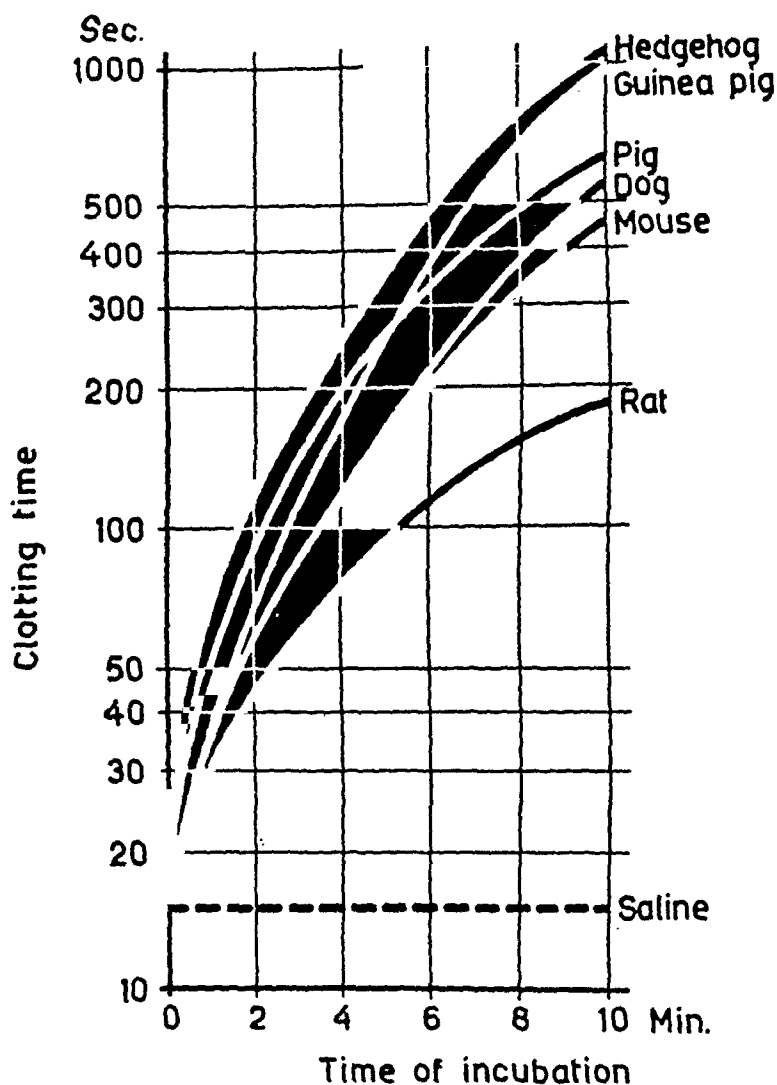


Fig. 2. Thrombin inactivating capacity in sera of group 2 of the species.

We have no explanation of this difference because the substances taking part in the inactivation of thrombin are unknown.

In a search for comparable data in the literature, I found those reported by SEEGER and SMITH (1942). They found that different amounts of thrombin are required to clot the plasma of different species in the same time. This phenomenon could be explained by means of the different thrombin-inactivating capacity of sera as shown above. The fact that human, rabbit and ox plasma required a lower (2.0—2.5 units) thrombin activity while

canine, pig and rat plasma required 5—10 thrombin units, agrees with the differences in the manner of inactivation in my experiments reported above.

### Summary.

1. Experiments carried out with low thrombin concentrations show that sera of different species under similar conditions inactivate different quantities of thrombin.

2. The course of the thrombin inactivation by sera of 12 mammalian species, during the initial phase, is calculated.

3. Sera of horse, cattle, rabbit, sheep and goat absorb thrombin, and the fermentative inactivation has a constant velocity during the initial phase. Another group of sera (rat, mice, dog, guinea-pig, pig, hedgehog) shows no absorption, the inactivation is more rapid, and the reaction velocity is not constant.

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AN EXPERIMENTAL STUDY  
ON  
AIR BLAST INJURIES

BY

CARL-JOHAN CLEMEDSON

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UPPSALA 1949





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## Introduction.

The term of "blast injury" is generally accepted nowadays as the indication of the multiplicity of changes, both patho-physiological and pathological, which occur within the organism when it is exposed to the effects of the pressure and impulse field around a detonating charge.

Blast injuries have probably existed ever since man learnt to use explosives. One of the first to mention injurious effects upon the organism caused by explosions was JARS (1768), who presented the theory that a miner in an exploding mine is injured by being exposed to a rapid compression and decompression, and that death is caused by "*la grande et prompte dilatation d'air*," an idea which, seen in the light of more recent experiences and research, must be regarded as essentially correct.

It is only in recent years, however, that blast injury has been more intimately known and taken notice of. The fact that explosion catastrophies could kill people without any apparent sign of external injury was indeed known from numerous great mine explosions (GAUDIN 1887, MITCHELL 1897, ZANGGER 1907, LLEWELLYN 1910, HATTON 1911, and others), and already during the first world-war soldiers were often found, after nearby grenade explosions, sitting or lying dead in the trenches without any kind of external injury whatsoever (MOTT 1916). The pathogenic connection between the shock wave set up by an explosion and these apparently unexplainable deaths was, however, still insufficiently analysed. Already JARS and later REYNAUD (1887) and others had indeed pointed out that the variations of pressure at the explosion were the cause of the injury, an opinion also supported by PAUL BERT (1878) in his classical studies of the decompression syndrome. This theory, however, was by no means generally accepted, and poisoning with the explosion gases, above all with carbon monoxide, was by many considered the most important factor (BROUARDEL 1887, CÉNAS 1887, DUJOL 1887, MITCHELL 1897, URIE 1904, BAHIER 1905, FABRE 1906, LLEWELLYN

1910, HATTON 1911, STIERLIN 1912, MOTT 1916, HILL 1917, LOGAN 1939, and HADFIELD *et coll.* 1940). Other authors maintained that some of the deaths caused by explosion could be explained by the existence of air emboli set free by the sudden decompression (GRANJON-ROZET 1887, GAUDIN 1887, GUINAND 1887, REYNAUD 1887, THOMAS 1917) a theory latterly supported by SCHÉR (1941), among others.

At the time before and during the first world-war the greatest stress was laid upon symptoms from the central nervous system (surveys by MAIRET and DURANTE 1919, KING and CURTIS 1942, and others), while, on the other hand, possible effects on other organs usually came to be quite overlooked. Great difficulties existed also in differentiating between what was actual organic lesion caused by an explosion ("shell concussion") and mere nervous manifestations due to fear and exhaustion. Many of the cases which nowadays would have been considered definite cases of blast injury were then probably referred to by the somewhat vague concept of "shell shock."

Some authors had already earlier pointed out that changes could occur in other organs than the central nervous system, in the lungs then above all (REYNAUD 1887, PAUCOT 1901, DELACROIX 1907, RAVAUT 1915, THOMAS and JOHNSON 1915, SENCERT 1915, among others), but the first to point out the dominating role played by the lung lesions in the syndrome of blast was the American physiologist HOOKER (1924). The investigations of recent years have shown even clearer the dominance of the lung injuries (see KING and CURTIS) and expressions such as "*blast lung*" (WILLIAMS 1942), "*blast chest*" (THOMAS 1941) and "*pulmonary concussion*" (OSBORN 1941) have been used, for excellent reasons, as closely synonymous with the concept of air blast injury.

Through the unexampled development of military technique in the last decade, blast injuries in modern warfare have become extremely common. The first real proofs hereof date from the Spanish Civil War when high explosives in projectile weapons, grenades and aerial bombs were used to an extent hitherto unheard of and whose technical and medical damaging effects are vouched for by HALDANE (1938), MOGENA (1938), LANGDON-DAVIES (1938), MITCHINER and COWELL (1939), KRETZSCHMAR (1940), and others.

Through extensive studies, both clinical and pathological, and also,

though in a comparatively less degree, through animal experiments especially in England and America during the last war, our knowledge of blast injuries has been considerably widened. The symptomatology and the pathological picture of ruptured ear drums, inter- and intra-alveolar haemorrhages and traumatic emphysema of the lungs, and in more severe cases meningeal haemorrhages and injuries to the abdomen in the form of haemorrhages and perforation of the intestines, therefore, are quite wellknown. On the other hand, many problems concerning the actual cause of the injury are still unsolved, and as to the factors which favour, or possibly restrain, the appearance of blast injury, our knowledge, in many respects, is still incomplete.

This applies even more to the effect of the blast wave on the physiological functions of the organism. Even RIEMBAULT (1883) and others realized that death due to blast was caused by changes of vital nerve functions, and MOTT (1916) pointed out that the respiration and circulation would be affected by the blast wave, and was of opinion that the enormous air pressure "may be transmitted to the base of the brain and cause shock to the vital centres of the floor of the fourth ventricle, causing instantaneous arrest of the function of the cardiac and respiratory centres." And HAMLIN (1943) points out that paralysis of the autonomic nervous system is one of the most outstanding physiological effects of the detonation causing an interruption of the reflex activity of circulation and respiration.

It is hoped that this work will contribute to some extent in throwing light upon a number of questions concerning the patho-physiological aspects of blast injury. Owing to the enormous size of the subject it has only been possible to bring up for discussion a few essential questions of which very little notice has been taken previously. These questions are:

1. The relation between the physical qualities of the blast wave (maximum pressure and impulse), and the extent of the blast injuries resulting from it, a problem, which, so far as it concerns the maximum pressure, has only been given slight consideration previously, and which, so far as it concerns the impulse, has hardly been mentioned at all. In this connection we will first touch upon questions concerning the development of the blast injuries and their localization to various organs. Only certain special problems will then be taken up for discussion, however, as these questions, especially where



the lung injuries and other pathologic changes are concerned, have been dealt with by other authors in a number of papers during the last ten years (see surveys by KING and CURTIS 1942, WILLIAMS 1942, TUNBRIDGE and WILSON 1943, THEIS 1943, BARROW and RHOADS 1944, DESAGA 1944, TUNBRIDGE 1945, COREY 1946, COHEN and BISKIND 1946, DRAEGER, BARR and SAGER 1946, and others).

2. The effect of the detonation upon the respiration.

3. The effect of the detonation upon the circulation organs and the blood circulation.

4. Changes in body temperature in blast injury.

5. The direct cause of death in blast injury. This question, which has been much discussed but is still unsolved, is, of course, to some extent, closely connected with those mentioned above. The question concerning the existence of traumatic shock in pure blast injuries and the haemodynamic and blood-chemical changes connected with it, is also taken up for discussion.

This paper only deals with air blast injuries. Immersion blast injuries, which in principle do not differ a great deal from these, have been discussed by the present author in an earlier paper (CLEMEDSON 1948). A number of surveys of this subject exist as well (see CAMERON, SHORT and WAKELEY 1942, 1943/44, AUSTER and WILLARD 1943, GOLIGHER, KING and SIMMONS 1943, GREAVES *et coll.* 1943, THEIS 1943, COREY 1946, and DRAEGER, BARR and SAGER 1946).

Finally, as regards the concept of "solid blast" recently introduced by BARR, DRAEGER and SAGER (1946) this embraces all the different injurious effects which are caused when the shock wave is transmitted to the organism through a solid object (armour plate, floor, ship's deck etc.). As these injuries are of a quite different nature to those caused by air blast they will not be discussed here.

## CHAPTER 1.

### The Detonation and the Physics of the Blast Wave.

ROBINSON (1944) gives the following definition of an *explosion*: "In a general sort of way, an explosion consists of the very sudden production of large quantities of gases, usually hot, from a much

smaller amount of a solid, a liquid, or a gas. This is usually accompanied by a loud noise or report." By *detonation* we generally mean the very sudden explosion of a high explosive.

When a solid high explosive detonates it is transformed, then, into glowing gas. This change begins in a certain point — the point of ignition — and continues along a boundary surface which rushes along in the explosive with a constant speed characteristic to every explosive — its detonation speed — and varies between 3000 and 8000 m/sec.<sup>1</sup> During this inner detonation, which only lasts some hundred thousandth of a second for a charge of approximately 1 kg. the reaction gases, which have been formed, are, consequently, within the original volume of the explosive. The temperature is then about 3000° C, and the pressure is of the order of  $10^5$  atmospheres.

Through the almost instantaneous transition from solid explosive to gas during very high pressure an enormous pressure gradient is obtained in the surface of the explosive. The gas expands perpendicularly outwards setting the surrounding air in motion. A great pressure increase, or shock front, will, therefore, spread out concentrically from the blast centre. The glowing ball of explosion gases holds together till it reaches approximately 10 000 times its original volume (PAYMAN and WOODHEAD 1937).

The shock wave propagates with a velocity which, nearest to the explosion centre, is several times greater than that of normal sound (defined as the velocity at fading excess pressure, see e. g. WEIBULL 1944).

In a given point some distance from the charge the pressure rises almost instantaneously up to a maximum — maximum pressure — and drops then more slowly down to atmospheric pressure, or below this (see fig. 1). The interval between the initial increase of pressure and the maximum pressure is for a high explosive, according to estimations, of the order of  $10^{-8}$  seconds. The shock-front therefore is very steep. According to estimations by PRANDTL (quoted from WEIBULL 1944) the depth of the shock front, that is to say, the distance between the foremost point of the wave and the position of the maximum pressure, is approximately 0.003 mm already at an excess pressure of only 0.04 kgf/cm<sup>2</sup>.<sup>1</sup> At higher pressure it is even less.

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<sup>1</sup> 1 kgf/cm<sup>2</sup> = 0.968 atm.

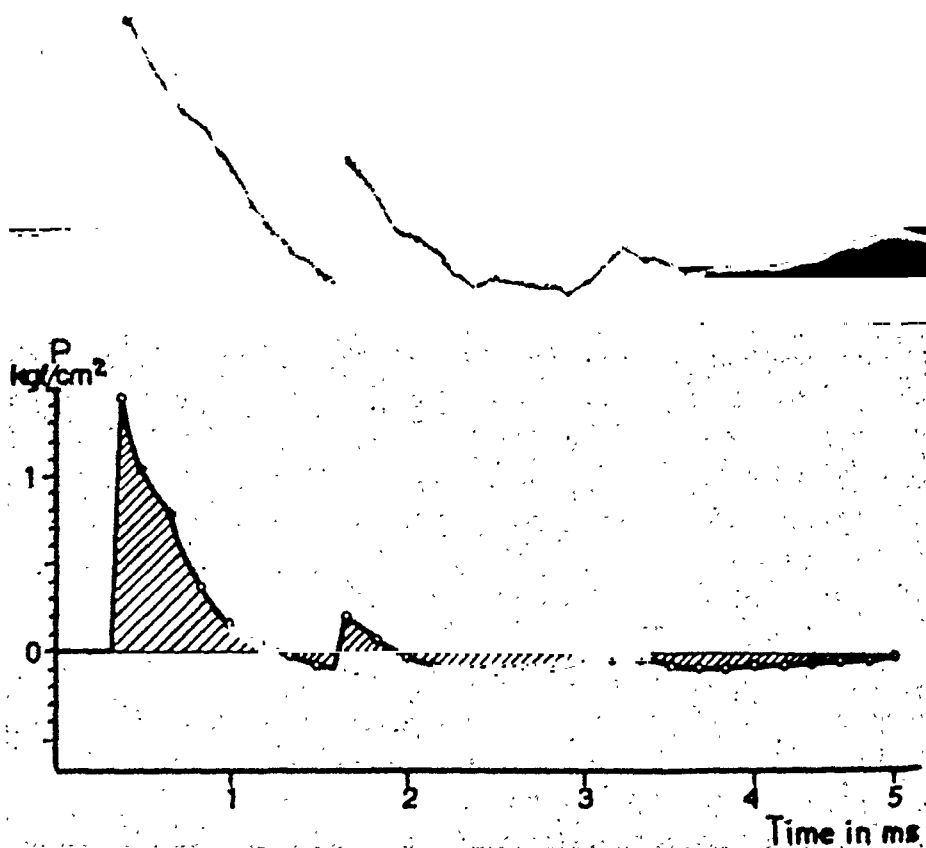


Fig. 1. The shock wave from a detonating charge of TNT. Weight of charge 0.10 kg. Distance from charge 1.16 m. *Upper curve*: Shock diagram recorded with the "Corona" microphone (described on page 26). *Lower curve*: Calibrated diagram of the same shock wave.

The duration of the pressure is somewhat different for different explosives and varies a little with the size of the charge and the distance from it. ZUCKERMAN (1940, 1941) states that the positive pressure wave at a distance of 10 metres from a charge of 32 kg lasts 5 or 6 milliseconds, and that the duration of the negative wave — the suction wave — is approximately 30 milliseconds. While the duration of the negative wave is fairly constant for one and the same explosive and weight of charge (ZUCKERMAN 1941, BERNAL 1941) the duration of the positive wave varies with the distance from the charge, owing to the fact that the positive phase of the shock wave always has greater speed than the following suction wave. The positive wave, therefore, will be more and more extended as the distance from the charge increases (see fig. 2).

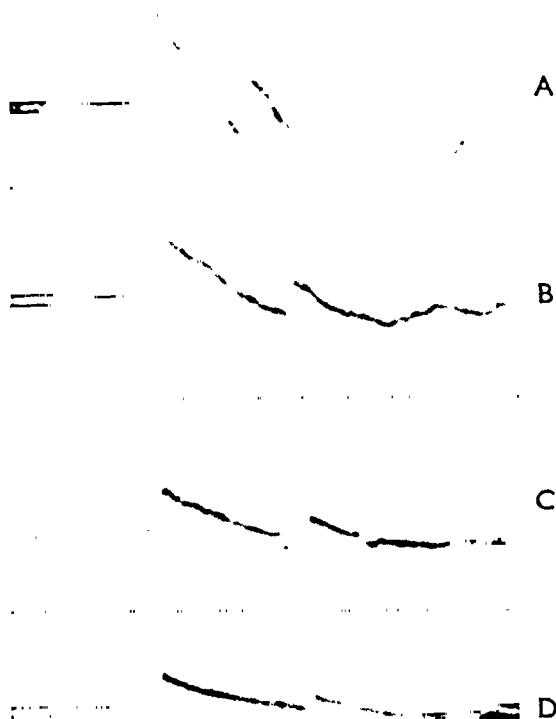


Fig. 2. The shock wave at different distances from a charge of TNT. Weight of charge 0.05 kg. Distance from charge in A = 1 m, in B = 1.5 m, in C = 2 m, and in D = 3 m.

Besides the positive and negative shock waves also a number of smaller oscillations occur at the detonation. These would have no physiological importance, however, (COREY 1946), and should be distinguished from the phenomena which occur in a closed space where reflexes and wall vibrations may cause standing waves.

In the boundary surface of an acoustically denser medium the shock wave is reflected with some slight divergence from the common laws of reflexion, owing to the intensity. The reflexion occurs without an appreciable phase displacement, but the reflected wave is always more or less deformed. — Cf. also SUTHERLAND (1940).

When investigating the various effects of the blast wave upon structures three principal factors should be considered, namely, the maximum pressure, the impulse and the energy of the shock wave. This paper will chiefly deal with the maximum pressure and the impulse, whereas the energy will be mentioned only by the way.

### The maximum pressure.

The maximum pressure ( $p$ ) is the highest pressure acting in a point passed by the shock wave from a detonating charge. This pressure consists partly of the pure hydrostatic pressure and partly of the pressure which is due to the movement of air in the vicinity of the charge. Close to the charge this latter pressure is as great as, or greater than, the hydrostatic pressure. The part of the pressure which is caused by the movement of gas, decreases further away from the charge, and at a relatively great distance it is quite negligible.

As most devices used in measuring the maximum pressure in shock waves, theoretically speaking, may be supposed to have a flat surface, it is worth mentioning here that the pressure which acts on these instruments is not identical with that which acts in a point. Owing, for instance, to the air being reflected by the flat surface, the pressure against this will be at least twice, and sometimes several times greater than that which strikes in a point.

The maximum pressure decreases rapidly with increasing distance from the charge. This decrease is not linear, however, as would be the case, if the pressure were transmitted through water. In fact the maximum pressure decreases with a potency ranging from 3 to 1 at short and great distances, respectively.

If the weight of charge is  $Q$  and the distance from the charge  $R$  the following formula applies for the maximum pressure with sufficient exactitude for TNT (trinitrotoluene), according to VON ZEIPPEL (1946):

$$p = 1.90 \cdot \frac{Q^{\frac{1}{3}}}{R} + 4.60 \cdot \frac{Q^{\frac{2}{3}}}{R^2} + 41.0 \cdot \frac{Q}{R^3}$$

At reflexion against a solid surface conditions are more complicated, and we may find one or several reflected waves, each with a maximum pressure of its own. If the charge is suspended above the ground, the shock wave is reflected from this in accordance with fig. 3. Even a delayed shock wave with the maximum pressure  $p'$ , which has been reflected by striking the ground and has covered the distance  $R'$ , will then act on a pressure gage in a point  $T$ , at height  $h$  and at distance  $R$  from the charge. Then the following approximate formula applies:

$$R' = R \left\{ 1 + \left( \frac{2h}{R} \right)^2 \right\}^{\frac{1}{2}}$$

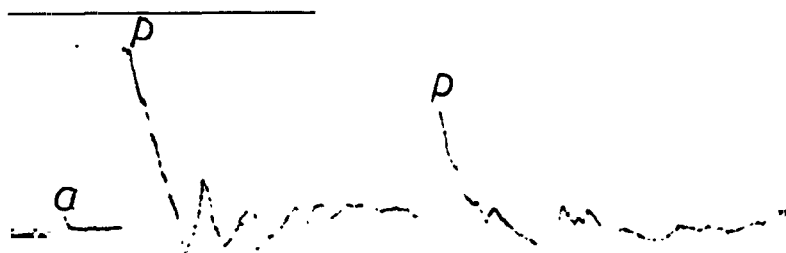


Fig. 3. The shock wave from a charge of TNT (0.10 kg) with reflexion from ground. *a*. Ignition of explosive. *p* = max. pressure. *p'* = peak pressure of the reflected wave.

and further

$$p' = \frac{p}{1 + \left(\frac{2h}{R}\right)^2}$$

Consequently *p'* is always lower than *p*. Moreover *p'* is further weakened by absorption at the reflexion.

The time difference ( $\Delta \tau$ ) between the arrival of *p* and *p'* at *T* which may have great physiological importance, is obtained from the following formula:

$$\Delta \tau = \frac{R}{\omega_1} \left\{ \sqrt{1 + \left(\frac{2h}{R}\right)^2} - 1 \right\}$$

where  $\omega_1$  is a mean value of the speed of the spherical shock wave ( $\omega_1 = \frac{dR}{dt}$ ).

### The impulse.

By impulse (*I*) we usually mean the time-integral of a force, in this case, therefore, the time-integral of the blast pressure (*p*) with the integration extended over the time it takes for the blast wave to pass:

$$I = \int_{t_0}^t p \cdot dt$$

The impulse is, consequently, the shadowed area in fig. 1. It is clear from this, then, that even if the pressure acting on a structure should change very rapidly, the impulse can, nevertheless, reach quite high values. Under certain conditions it seems plausible that the impulse has greater importance in producing injury than the maximum pressure.

The following formula applies to the impulse:

$$I = C \cdot \frac{Q}{R^2}$$

$C$  is a constant.

This formula is valid at moderate distances from the charge.

### **The energy of the shock wave.**

The energy of the shock wave consists of two different components, namely, a potential component manifested as the pressure in the elastic medium (air) and a kinetic component represented by the speed of particles in the medium.

### **The principle of similitude (the scaling law).**

For practical purposes it has been found convenient always to refer the action of a detonating charge to that obtained from a charge of 1 kg (unit charge).

In calculating the effect, one then uses the scaled distance obtained from the following formula:

$$r = \frac{R}{\sqrt[3]{Q}},$$

where  $r$  = the scaled distance,  $R$  = the actual distance, and  $Q$  = the weight of charge in kg.

At distance  $r$  from a unit charge one gets the same effects as at distance  $R$  from a charge of  $Q$  kg.

## **CHAPTER 2.**

### **Earlier Animal Investigations.**

Despite the existence of no doubt important differences between the human being and various animal species as regards their sensitivity to the effect of blast waves, animal experiments doubtless offer far greater possibilities in the study of detonation effects than clinical observations, owing to their more systematic and controllable conditions. In consideration hereof it is remarkable how few experimental investigations actually have been made in this interesting, and from a military-medical aspect, very important domain.

As far as the literature is concerned, the earliest animal experiments as to the effect of the blast wave on the organism were made no earlier than during the first world war. The experiences from the Balcan war then caused RUSCA (1914) to examine on rabbits "die traumatischen Druckwirkungen der Explosion." He was then able to show that animals were killed if exposed at a short distance from a detonating charge, and that the area within which this fatal effect was caused was almost 10 times greater in water than in air. CRILE (1917) exposed rabbits to explosions in confined spaces and found massive haemorrhages in the lungs. Similar pulmonary lesions as well as haemorrhages, microscopic ones in particular, in different parts of the central nervous system were noted by MARINESCO (1918) in his blasting experiments with small charges of "fulmicoton" against dogs. Haemorrhages in the liver, spleen, kidneys and adrenals could occur, but were less common. Blood was observed in the nose and mouth of one animal, and others suffered from dyspnoea, depression and walking difficulties.

In experiments by MAIRET and DURANTE (1917, 1919) the essential pathological findings were patches or confluent zones of haemorrhages in the lungs, also small haemorrhages on the surface of the spinal marrow as well as inside it, and around the nerve roots. In three separate series of experiments they exposed rabbits to the shock wave from small charges of *melinite* (picric acid and collodium) and *cheddite* (a chlorate explosive). The animals were first exposed at a distance of 1—1.5 m from a charge and at the same height as this, secondly, in order to find the effect caused at different angles of incidence ( $20^{\circ}$ — $50^{\circ}$ ) they were suspended at a distance of 2.5 m from a charge on the ground, and thirdly they were arranged in different positions in trenches. The authors submitted their material to fairly thorough examination from a pathological point of view, especially concerning the central nervous system. They emphasize the discrepancy between the animal's general condition immediately after the detonation and the degree of injury manifested by its further development during the next 24 hours. As to the pathogenesis they present several interesting points of view, for instance, that the extent of the injury to a certain degree depends upon the amount of blood in the blood vessels. It would doubtless be of some importance, therefore, whether the vessels were in systole or diastole, whether the individuals were congestive or anaemic and had a high or low blood



pressure, and whether the lungs were in an inspiratory or expiratory position. They point out, finally, that the blast wave affects such parts of the organism above all where the blood vessels are inadequately supported by surrounding tissue as the case is, for instance, with the lung capillaries.

CARVER and DINSLEY (1919), on the basis of their blast experiments with small charges of geglinite with rats and mice and in immersion experiments with fish as experimental animals, divide the region around a detonating charge into three different zones. In zone A ("*zone of brisance*") which consists of the region in which the detonation causes a crater, animals are torn to pieces or obtain severe internal injuries with haemorrhages in different viscera, the central nervous system and the eyes, and often bleed from the nose and mouth. In zone B ("*zone of decompression*") animals are killed often without external injuries but with severe lesions in different viscera. This zone merges into zone C where the effect on the animals varies a great deal, frequently, however, consisting of a transitory stupor, followed by a more or less marked excitation.

HOOKE (1919, 1924) in the winter of 1918—1919 carried out some investigations chiefly in order to establish the existence of "primary shock" in "air concussion." He states that lung rupture and lung haemorrhages were "the single gross lesion found post-mortem in cats, dogs and rabbits after exposure to air concussion due to gun blast or high explosive." No relation seemed to exist between the extent of the lung injury, the animal's general condition and the degree of primary shock. HOOKE is of opinion that the shock caused by air concussion is of a different nature to the forms of shock known at that time. The fact that primary shock occurred in exposure before a gun muzzle but not in experiments with TNT, though the maximum pressure acting on the animal was practically the same in both cases, indicated, he thought, that it is the duration of excess pressure rather than its amplitude that is the deciding factor.

In experiments with sectional steel shelters in England just before the second world war BARCROFT (1939) carried out blasting experiments with goats and rabbits in the field and in shelters and was able to show, that shelters gave a certain degree of protection against the blast wave and that the animals closest to the charge and exposed to high positive as well as negative pressure, chiefly had lung injuries.

Investigations by ZUCKERMAN (1940, 1941) and KROHN, WHIT-

TERIDGE and ZUCKERMAN (1942) are the chief experimental contribution to our present knowledge of the effect of blast waves upon the organism. Extensive experiments were made where rats, mice, guinea pigs, rabbits, cats, monkeys and pigeons were exposed to detonations from TNT charges of about 35 kg and compounds of hydrogen-oxygen gas in balloons, and where the pressure and form of the blast wave were registered with piezo-electric gauges. ZUCKERMAN found then that if animals were protected from splinters and were injured by blast only, the extent of the injury depended on the kind of animal used and on its distance from the charge, in other words, on the blast pressure. Animals, who had not been close enough to the charge to be torn to pieces, showed various degrees of lung lesion ranging from a few dots of blood on the surface or haemorrhages here and there in the whole lung parenchyma and to complete hepatization of whole lung lobes. In moderate cases the middle and lower parts of the lung in particular were affected and especially its anterior borders. In 40 per cent of the animals haemorrhages were also found in different abdominal organs, and in some single cases in other internal organs as well. Animals with a protective garment covering that part of the chest which faced the charge received little or no lung lesion. The fact that animals very close to the charge received injuries mainly on that part of the body which faced the charge proves, ZUCKERMAN considers, that the blast wave causes injury by "the impact of the pressure component of the blast wave on the body wall" and not, as often previously supposed, by passing downwards via the air passages in which case both lungs should have been injured. These observations have been pursued further by KROHN, WHITTERIDGE and ZUCKERMAN (1942) who have also studied the effect of blast waves upon respiration, circulation and cerebral activity.

In contrast to other authors, COREY (1946) believes that investigations of air blast injuries have no great practical importance. He then bases his opinion on experiments carried out in the U. S. Navy which have shown that only those animals which had been within the "flash area" and at the same time had received burns, had been injured by the detonation. In virtue of blast experiments on goats HORWATH and SHELLEY (1946) like COREY, consider air blast relatively harmless to living creatures. They showed by blast experiments in a tunnel, on the other hand, that the effect is considerably

greater in confined spaces. Thus, animals placed in a field at exactly the same relative distances from a charge similar to that used in the tunnel experiment, received much slighter injuries. In the mortally injured animals there were apart from lung injuries, also haemoperitoneum, haemorrhages in the diaphragm, lacerations of the liver and ruptures of the stomach. In addition there were also mediastinal emphysema and air bubbles in the pericardium, an occurrence never previously found in blast experiments.

### **Earlier investigations of the effects of blast waves on certain physiological functions.**

With the exception of occasional observations, especially concerning changes of the respiratory rate caused by the detonation, only HOOKER (1919, 1924) and KROHN, WHITTERIDGE and ZUCKERMAN (1942) have studied some of the physiological functions of the organism after exposure to blast. Their investigations of the circulation and respiration of animals injured by blast have actively contributed to the understanding of certain aspects of injurious effects due to blast, but are, at the same time, incomplete. These investigations will be discussed later.

In this connection an investigation by CLARK and WARD (1943) is worth mentioning. They tried to imitate on a small scale a blast wave in water, and observed changes of respiration and heart action in rats and cats exposed to this shock wave.

Certain attempts have been made to imitate blast effects in the laboratory. Thus YOUNG (1945) imitated "in slow motion" the pressure of the blast wave upon the organism. He fixed firm strips of canvas round thorax and abdomen of animals, and registered the changes in the intra-pulmonary pressure and in the spinal fluid, and also in the arterial and venous pressures, when these strips were tightened.

It should, perhaps, be emphasized already here, that these investigations have greatly contributed to the understanding of the tolerance to compressive stress of the cardiovascular and respiratory systems, but all the same, we must not draw too far-reaching conclusions from these investigations as to the effects caused by the detonation. The fact is that both earlier investigations (HOOKER 1924, and others) and those mentioned below show that the reac-

tions of the circulation and respiration to a large extent depend on the rapidity of the increase of pressure and its duration. Similar reflections apply to experiments by WHITEHORN, LEIN and EDELMAN (1946) and EDELMAN, WHITEHORN, LEIN and HITCHCOCK (1946) who have examined the tolerance of the circulation towards explosive decompression. Only the influence of a rapid reduction of pressure is studied here. However, the positive phase of the wave, i. e. the pressure increase, surely plays at least as great a part in blast as the suction wave (cf. ZUCKERMAN, 1940, 1941, and others).

### CHAPTER 3.

## Theories Concerning the Mechanism of Blast Injuries.

When an organism is struck by a shock wave the incident wave is reflected in accordance with what was mentioned on page 13. Due to its being dammed up and reflected the pressure in the boundary surface itself becomes greater than in the incident wave. Some of its energy, however, will probably be transmitted through the tissues and will pass through these like a shock wave, though with almost similar speed to that of sound in water. How much of the pressure is reflected and how much is absorbed in the boundary surface or passes through the tissues, is still quite unknown.

Concerning the mechanism of lung lesions due to blast three main theories have been suggested:

1. The negative shock wave, or suction wave, leads to rupture of the lung capillaries. (LOGAN 1939, SHIRLAW 1940.)
2. The lungs are suddenly expanded with air which leads to rupture of the lung tissues. (BARCROFT 1939.)
3. The lesions are caused by direct impact when the chest is struck by the steep shock front. (ZUCKERMAN 1940.)

The first theory espoused by LOGAN and others seems supported by investigations by LATNER (1942) and EDELMAN *et coll.* (1946), among others, who have studied the effect of explosive decompression and so produced lung injuries similar to those in blast injury. Experiments by CARLTON, RASMUSSEN and ADAMS (1945) in which lung haemorrhages and coronary air emboli were obtained when air

was forced into trachea during moderate pressure increase (35—240 mm Hg), seem, to some extent, to support BARCROFT's idea. For either of these two theories to be accurate, however, it must be assumed that the shock wave can pass down through trachea. ZUCKERMAN (1940), however, seems to have shown without any doubt that this does not happen in any degree worth mentioning, and his theory would probably be the one generally recognized to-day.

The lung lesion would, therefore, be assumed to result from the severe contusion and compression of thorax caused by the phase of high pressure. To this effect we must then add the effect produced by the part of energy which is transmitted through the organism, as in the boundary surface between acoustically different media — the boundary between tissue and air — an expulsion effect occurs, and the tissues are torn to pieces. This is the reason why organs containing air or gas in particular, are injured, while changes in muscles and solid organs are proportionally rare and only occur at very high pressure.

Abdominal injuries are proportionally less common in air blast and such viscera which contain gas are mainly affected. The same reflections would apply concerning the mechanism of intestinal haemorrhages and perforations in air blast as those made by GREAVES *et coll.* (1943) concerning these changes in immersion blast, namely, that "if the gas can be displaced without compression a haemorrhagic lesion of the wall will be the only result. Perforation will occur when the gas bubble is trapped and becomes compressed under the pressure of the wave and snaps back in re-expansion when the wave passes."

The share in the blast effect of the different physical components of the blast wave is still very little analysed. The extent of the lesion would not be determined by maximum pressure alone, but, as already emphasized by HOOKER (1924) also by the duration of the pressure increase. Even the impulse, therefore, would be of importance. As regards this question WAKELEY (1945), in an interesting survey of the effect of immersion blast on the human organism, calls attention to the fact that it is neither the maximum pressure nor the impulse alone which determines the extent of the lesion. A high maximum pressure by itself is not sufficient to cause lesion unless it lasts long enough for the tissues to lose their power of resilience, and the breaking-point is reached. On the other hand, a great im-

pulse by itself causes no lesion unless the pressure at the same time is so high that the structure fails to resist it, and breaks.

## *Experimental Investigations.*

### CHAPTER 4.

#### **Common Experimental Arrangements.**

These investigations have been carried out in experiments with high explosives both in the field and in a detonation chamber specially constructed for the purpose.

*Animals.* Rabbits of common country stock have mainly been used. All animals, both for experiments and control, have been taken from the same stock. They have been fed on hay, oats and water before, as well as after the experiments.

Rabbits have been chosen chiefly because they can be obtained in large numbers, are relatively cheap, and are easy to work with in serial experiments. The total number of animals used is approximately 350.

*Anaesthetics.* Urethane has been used in doses of 1.4—1.6 g per kg body weight given in a 20 per cent water solution. When anaesthesia of only short duration has been needed Narcotal<sup>1</sup> (isopropyl- $\beta$ -bromallyl-N-methyl-malonycarbamid-sodium) has been given in some cases instead of urethane. It has been administered in slow intravenous injections of 0.5—1.5 ml of a 5 per cent solution.

All the animals on which surgical treatment of some kind has been carried out, and all the remaining animals with the exception of 110, have been anaesthetized.

#### **Experimental arrangements in field blastings.**

In these experiments two different ways of grouping the animals and measuring instruments have been used, one in blastings with small charges, the other in blastings with charges above 2 kg.

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<sup>1</sup> Made by Astra, Södertälje, Sweden.

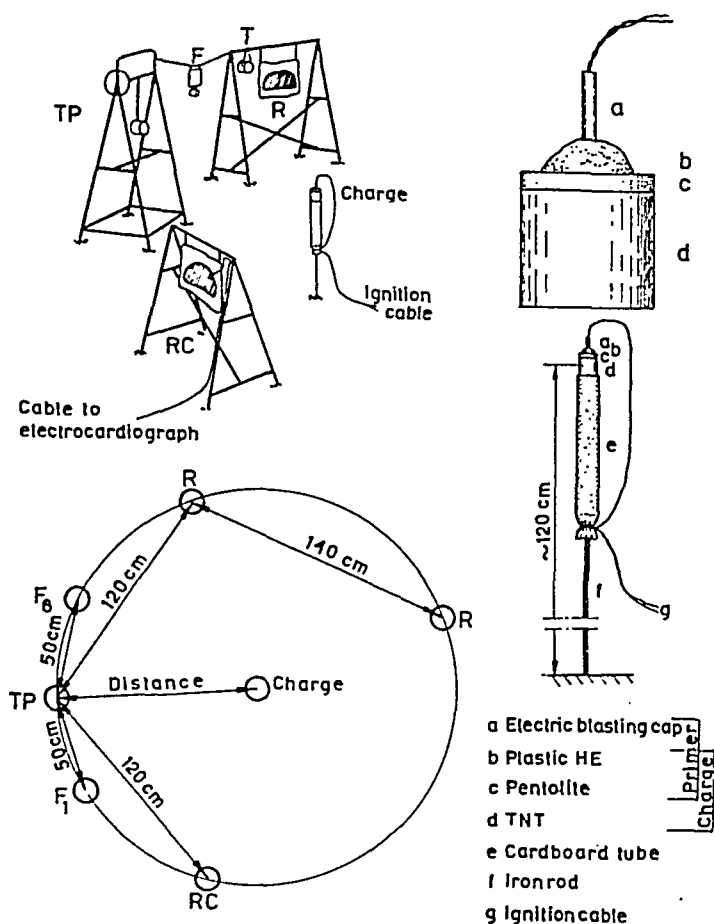


Fig. 4. Grouping in experiments with small charges. Left upper figure shows the stands supporting pressure and impulse gauges (T, TP and F) and animal cages (R and RC). Left lower figure: plan of the grouping arrangements. Right: the charge.

#### a. Grouping in blastings with smaller charges ("group type A").

In experiments with "group type A" the blasting area was a smooth sand field approximately  $25 \times 25$  m encircled by thinly scattered trees. The animals and measuring instruments were placed in a circle around an iron rod which had been driven into the ground in the centre of the field, and on which the charge was placed (see fig. 4). The animals were suspended in narrow wire mesh cages in stands similar to those which supported the pressure and impulse gauges described below. The cages were fastened with their top edges to the stand so that the animals hung about 120 cm from the ground

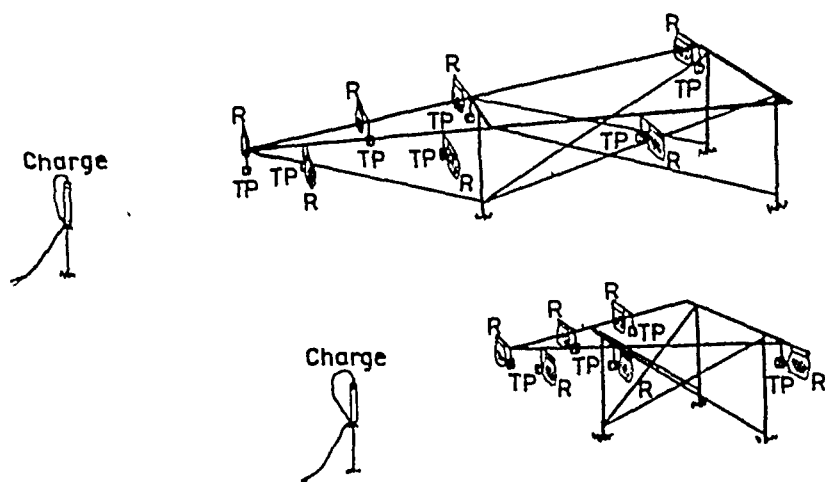


Fig. 5. Grouping in experiments with big charges. Wooden stands supporting pressure and impulse gauges (TP) and animal cages (R).

and level with the gauge and charge. Experiments with this grouping comprise 69 blasts with 125 rabbits in all.

#### b. Grouping in blastings with bigger charges ("group type B").

In experiments with "group type B" the blasting area was a fairly smooth field with a radius of about 50 m and with open country on three sides. The gauges and cages were suspended in large, heavy, V-shaped wooden stands 3—5 m long and 180 cm high (see fig. 5). Due to the stands being shaped like a V the animals did not shield one another or any of the gauges. Experiments with this grouping comprise 13 blasts with 72 rabbits in all.

In all experiments the rabbits were placed with their right sides towards the charge. The distance of the animal from the charge was always measured from the right side of the chest wall to the centre of the charge. The distance of the rabbits from the charge corresponded to that of the gauges from the charge (measured to the centre of the gauge membrane) with a difference of  $\pm 1$  cm in "group type A" and  $\pm 5$  cm in "group type B."

#### The charge.

In field blastings cylindrical charges of cast TNT weighing from 0.2—30 kg have been used. Greater weights of charge, up to 200 kg,



have been obtained by combining several cylinders. In experiments with "group type A" the weight of charge has varied between 0.230 and 1.150 kg, and the distance of the animals from the charge has been between 1.0 and 3.0 m. In experiments with "group type B" charges have been used weighing from 1.25—200 kg, and the distance of the animals from the charge has varied between 1.34 and 22.52 m.

The charges have been ignited by an electric blasting cap. In most cases a smaller charge of pentolite and a plastic explosive have been used as primer.

Owing to the cylindrical shape of the charge the blast field will diverge quite considerably in certain directions from spherical symmetry. This can be avoided to some extent by using spherical charges of pressed TNT. Charges of this type have not been available, however, and cylinder-shaped charges have been used instead. This disadvantage has been eliminated, as far as possible, by grouping the animals and gauges uniformly. They have always been placed in the equatorial plain of the cylinder and level with the charge.

## CHAPTER 5.

### Methods of Analysis.

#### A. Physical methods.

##### 1. Recording of the shock wave.

In recording the shock wave of a detonation several different methods have been used of which the piezo-electric is one of the most popular. This has several disadvantages, however, for instance, the sensibility of the piezo-electric crystals to various outside interference and the difficulty in obtaining recordings in several different points in one and the same experiment owing to the complicated construction of the recording devices.

In recording the shock wave both in field blasts and in the detonation chamber the "Corona" microphone, invented by CRIBORN (1947), has been used here (see fig. 6). This is based on the fact that in a point discharge between two electrodes with a high voltage the intensity of the current varies in accordance with the air pressure. The microphone, which, in principle, consists of an annular positive electrode in whose centre is a pointed negative electrode connected to the negative pole of a high tension unit (approximately 4 kV), is connected to a cathode ray oscillograph. The process is recorded on a film with the constant speed of 1 mm per  $10^{-4}$  sec.

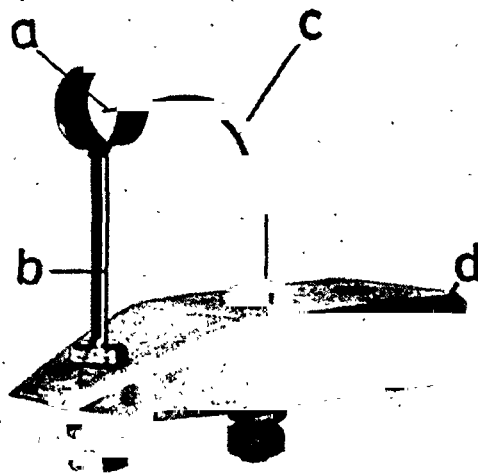


Fig. 6. The "Corona" microphone. *a.* platinum point, *b.* pos. electrode, *c.* neg. electrode, *d.* holder of plexiglas (lucite).

## 2. Determination of maximum pressure.

Of the many different instruments constructed for the recording of pressure only a few are suitable for measuring the maximum pressure of a blast wave.

ZUCKERMAN (1940, 1941), KROHN, WHITTERIDGE and ZUCKERMAN (1942), and others have used piezo-electric gauges in their investigations of blast injuries. American scientists have used several different types of mechanical gauges where the pressure acts upon a piston or membrane (COREY, 1946). An example of the piston principle is the "William gauge" which consists of a brass cylinder where a piston, when influenced by a pressure, indicates a movement which is proportional to the maximum pressure.

Membrane gauges of different types exist, for instance, those which determine the pressure by deflection of the membrane, and those which have a number of membranes with different diameter and thickness which break at different loads of pressure.

A membrane gauge of the first type is the Baroscope invented by MILLER (1919) and used by HOOKER (1924) in his studies of blast injury.

A critical examination of different recording methods shows that piezo-electric methods are less suitable due to the reasons already mentioned. The first type of membrane gauges seems to give the safest results.

In these investigations a considerably improved modification of MILLER's gauge, made by VON ZEIPPEL (1944, 1946) has been used. This (see fig. 7) consists, in principle, of a membrane of duralumin with a diameter of 50 mm. When a blast wave strikes the membrane it presses in a small friction-braked needle whose friction has been chosen great enough for it to stop even at the

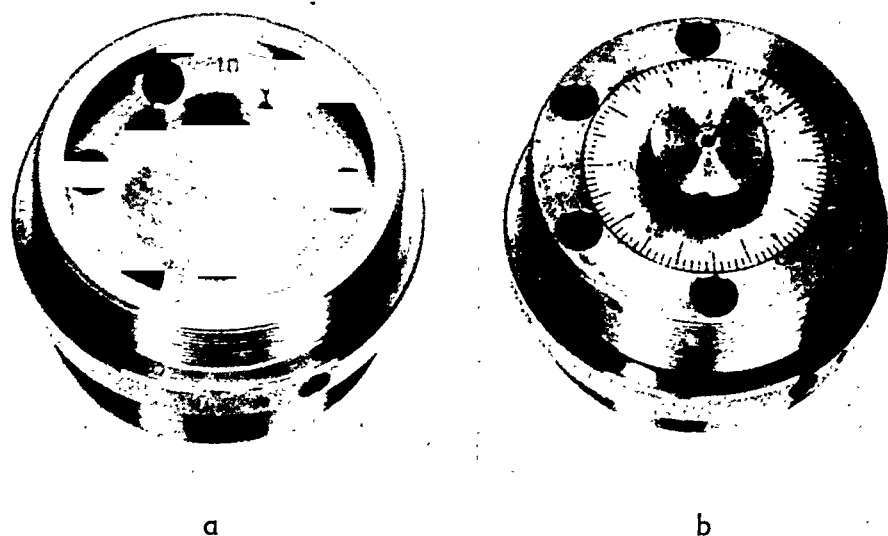


Fig. 7. The maximum pressure gauge. *a*. The front of the gauge showing the membrane. *b*. The back of the gauge with the micrometer screw.

highest pressures which the gauge can stand. In this way the deflection of the membrane can be recorded within a thousandth of a millimeter by a micrometer screw on the gauge. The thickness of the membrane of the different gauges varies from 0.5—4 mm with a natural frequency of from 2500—15 000 hz.

The gauges are statically calibrated at  $+19^{\circ}\text{C}$ . The calibration curve varies with the temperature with approximately 0.5 per cent per degree, why correction for this should be made. Some other corrections have been necessary. For instance, it has been proved an advantage to correct the values obtained from gauges with thinner membranes to values obtained from gauges with a 4 mm membrane (natural frequency = 15 000 hz). It has also been found (VON ZEIPPEL 1946) that a certain reading obtained when the membrane was struck by a blast wave did not correspond to that pressure which gave a similar reading at the static calibration. The reason for this is that the membrane is so slightly damped that a shock pressure will cause an excess deflection of the membrane of almost 100 per cent. For a gauge with a natural frequency of 15 000 hz the actual pressure, therefore, is only about 52 per cent of what has been recorded.

The uncertainty of the pressure values will be discussed further in chapter 7.

### 3. Determination of impulse.

The impulse of a blast wave may be determined in two principally different ways, namely either by estimating it from a recorded pressure-time curve, for instance, a piezogram, or from the additional speed given to a body within the impulse area. The latter method is probably the most popular, and has been used by WEIBULL (1944) and VON ZEIPPEL (1945), among others.

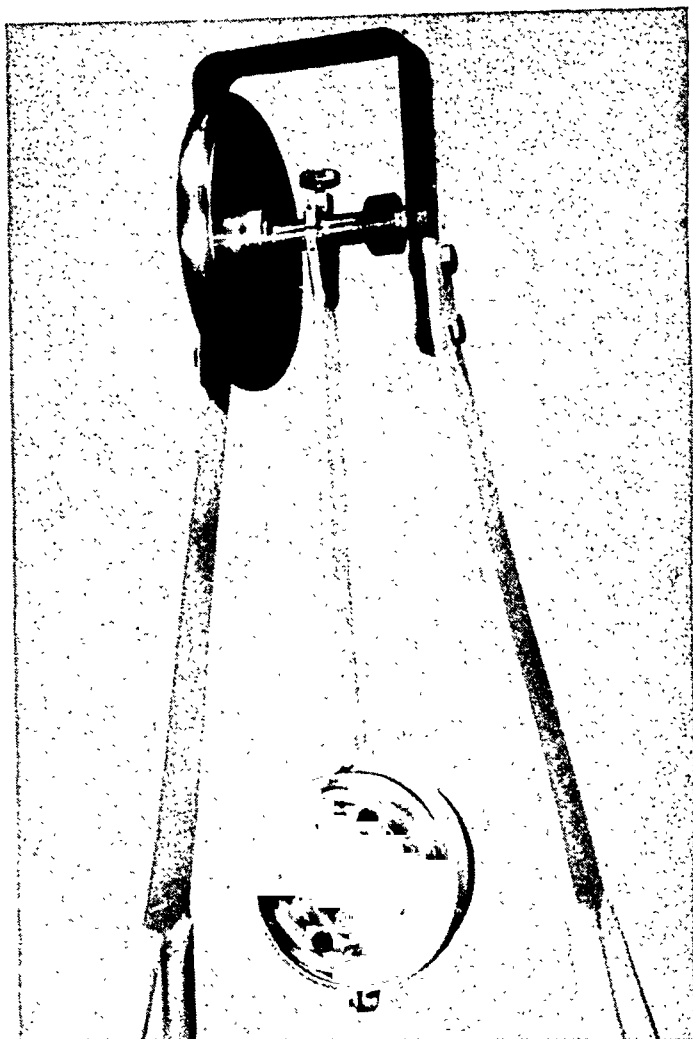


Fig. 8. The impulse pendulum.

In these experiments two different methods have been used for measuring the impulse by mechanical means. They have been elaborated by VON ZEIPPEL (1945) and are the impulse pendulum method and the "drop cylinder" method. The impulse pendulum (fig. 8) is a ballistic pendulum whose impulse receiver, for the main part, consists of the pressure gauge, described above. The pendulum is suspended on ball-bearings with very small friction. Its swing is recorded on a graduated disc by a needle which follows the movement of the pendulum and stops at maximum amplitude of the swing.

If excluding the air resistance and other outside disturbances the following formula (VON ZEIPPEL) applies to the impulse ( $I$ ):

$$I = \frac{m \cdot k}{g \cdot O \cdot L} \cdot \sqrt{2 g \cdot l (1 - \cos \varphi) + \frac{2 K \varphi}{m}} \text{ gf. sec/cm}^2{}^1,$$

<sup>1</sup> 1 gf.sec/cm<sup>2</sup> = 981 dyn. sec/cm<sup>2</sup> (g/cm.sec.).



Fig. 9. The "drop cylinder."

where

$m$  = mass of pendulum, in g.

$k$  = radius of inertia of pendulum.

$g$  = 981 cm/sec<sup>2</sup>.

$O$  = front surface of pendulum, in cm<sup>2</sup>.

$L$  = C. G.<sup>1</sup> distance of frontal surface of pendulum from axis, in cm.

$l$  = C. G. distance of pendulum from axis, in cm.

$\varphi$  = maximum angle of deflection of pendulum, in radians.

$K$  = friction moment of pendulum, in dyn. cm.

In order to increase the sensibility and decrease the error of the impulse value when measuring small impulses a collar of masonite has been fixed around the receiving part of the pendulum. Its surface has thereby become 10 times larger.

The "drop cylinder" (fig. 9) consists of a short metal cylinder suspended from two points with one end surface turned towards the charge, and so lightly that it becomes immediately unhooked by the shock wave. The impulse of the shock wave throws the cylinder a distance of  $l$  cm, whereby it falls  $h$  cm from its suspension point to the ground. The impulse is obtained from the formula:

$$I = \frac{m \cdot l}{\sqrt{2g \cdot h}}$$

where

$m$  = mass per cm<sup>2</sup> of the end surface of the cylinder.

<sup>1</sup> C. G. = centre of gravity.

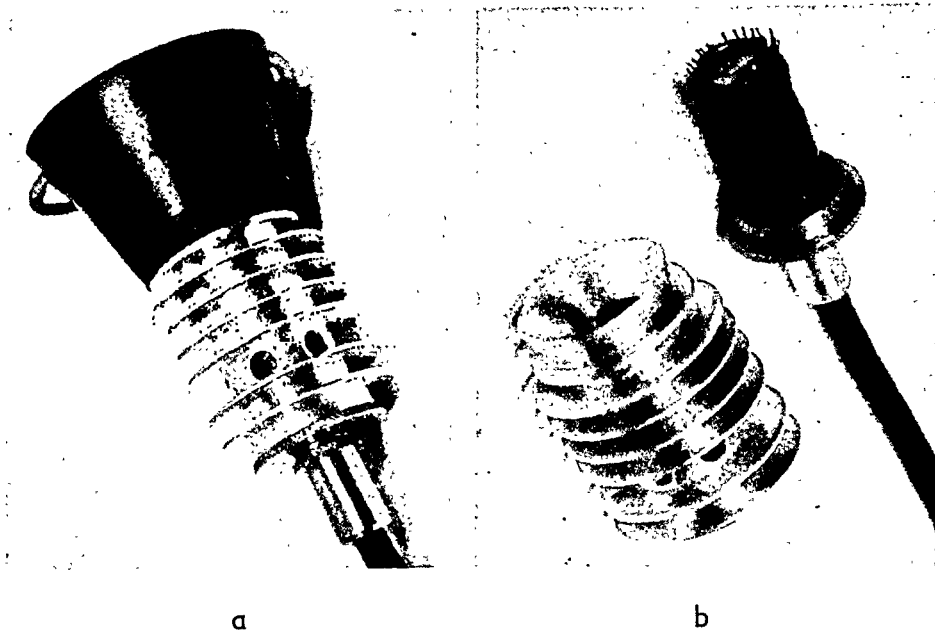


Fig. 10. The thermoelectric respiration recorder. *a*. The thermo gauge with metal mantle and rubber mask. *b*. Mask and mantle removed to show the thermocouples.

Two cylinders (F 1 and F 6 in fig. 4) have been used, their mass/cm<sup>2</sup> being 3.2 and 24.4 g and their measuring ranges 0.006—2 and 0.05—14 gf.sec/cm<sup>2</sup>, respectively. The cylinders were suspended as shown in fig. 9. They were allowed to fall upon a flat piece of ground covered with fine sand where the vertical line from the suspended cylinder had been marked beforehand by a small iron peg. The values obtained with the two cylinders always agreed.

As to the uncertainty of the methods see chapter 7.

## B. Physiological methods.

### Respiration.

Respiration recordings have been made continuously before, during and after the detonation. Owing to the special nature of the experiments, i. e. the very rapid and great variations of pressure, it has not been possible to use any of the methods otherwise employed by which the respiratory movements are passed to a recording instrument by means of air- or liquid transmission.

The present author, therefore, has improved and used the principle for respiration recording previously applied by BIERMAN (1946) which is based on a thermo-electric recording of the temperature difference between the inspired and expired air (CLEMEDSON and PETTERSSON 1947). The apparatus consists of a thermogage (fig. 10) connected to the fourth channel of the electrocardiograph (Triplex, Elmqvist's principle) which has been used for simultaneous recording of heart action.

The thermogage consists of 20 thermocouples connected in series and made of nickel-chromium constantan. These are fastened round a thin aluminium cylinder (fig. 10 b) and isolated in such a way that the junctions of one end only are free to be flushed by the respiratory air. Covering the cylinder is a metal container fixed on with screws for reducing disturbances caused by electricity and heat.

The thermogage is fastened in front of the animal's nose by means of a conical rubber mask kept in position with elastic tape.

The apparatus gives good respiratory recordings, except during the first tenth parts of a second after the detonation when a distortion independent of the respiration is seen.

### Heart action.

This has been recorded with an electrocardiograph using the three common extremity leads. The choice of electrodes is important as outside interferences must be avoided as far as possible. In earlier investigations subcutaneous needle electrodes have been used with preference, but also other types of electrodes have been employed. Thin zinc sheets have, thus, been used by STRAUB (1909), grooved amalgamated zinc sheets by AGDUHR and STENSTRÖM (1930) and silver sheets by MAYEDA (1934). KROHN, WHITTERIDGE and ZUCKERMAN, who have recorded the heart action in blast experiments, have led off from right shoulder and left thigh by means of subcutaneously placed silver-foil electrodes. STOCKER (1940), finally, has used electrodes inserted in the middle part of oesophagus and in rectum.

Extremity leads with subcutaneous needle electrodes have been used successfully in animal experiments by VERNEY (1924), MOSLER and SACHS (1925), SCHINZEL (1933), SCHIRRMESTER (1939) and WIZER and HABÁN (1939), among others. LEPESCHKIN (1942) points out that ordinary injection needles can be used, but that the part inserted under the skin must be at least 4 cm long.

In this investigation ordinary injection needles have been used with a diameter of approximately 1 mm and at least 4 cm long. They were inserted subcutaneously and firmly fixed with plaster round the leg and connected to the channels of the cardiograph by means of a 16 m long screened cable.

The electrocardiograph was placed in a shelter of reinforced concrete, situated about 10 m. from the blasting center.

*As regards animals where E. C. G. were taken at different times after the detonation special care was taken always to keep the animal in exactly the same position at the different recordings.* In experiments with unanaesthetized animals and in blastings in the detonation chamber, the animals were exposed in a sitting posture in the cages. In all other cases they were lying stretched out on their right sides.

### Blood pressure.

The arterial blood pressure has been determined by two different methods. Immediately before, during and after the detonation the pressure in the car-

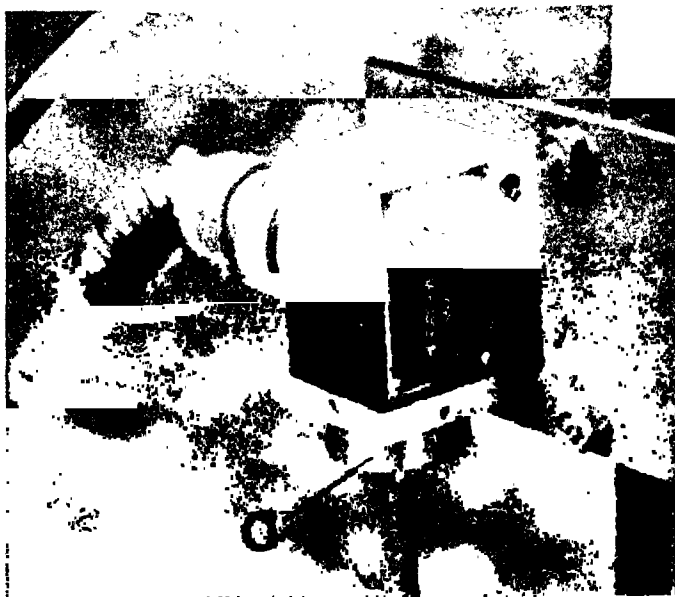


Fig. 11. The strain gage element of the blood-pressure recorder. *a*. Pressure chamber with the tube connected to the carotid artery cannula.

tid or femoral artery has been recorded by the bloody method. In animals where the blood-pressure has been followed for a longer period, say some 48 hours, a bloodless method has been found more suitable.

A survey of the different principles and methods of determining the blood-pressure in small animals has been given by LITHANDER (1945) who has developed and used the method given by FAHR (1938—39) for bloodless determination of the pressure in the central artery of the rabbit's ear.

Similar methods have been applied with success by VON RECKLINGHAUSEN (1906), ANDERSON (1922—23), KURAYA (1923—24), WATSON (1926), SQUIER (1927—28), SCHMIDT-WEYLAND (1931—32), GRANT and ROTSCCHILD (1934), GRANT (1935) and DOWNMAN, MACKENZIE and McSWINEY (1944), and others.

*The bloody method.* For recording the changes in blood-pressure occurring immediately after the detonation an apparatus has been invented (CLEMEDSON and PETTERSSON 1948), which is based on the strain-gage principle, that is to say, it contains a fine resistance wire which, when subjected to variations of pressure, alters its electric resistance.

The blood-pressure recorder (fig. 11) consists of a strain-gage measuring body (Stratham Gage Cat. YE-48-60 Ser. 389, Stratham Laboratories, Los Angeles), which has been provided with a small pressure chamber with a 0.05 mm thick phosphorus bronze membrane, that influences the tension of the measuring wire by way of a metal needle. By means of a flexible copper tube with delicate lumen the pressure chamber is connected to a cannula which is inserted in the central part of one of the exposed common carotid arteries or, in experiments with sinus denervated animals, in the right femoral artery.



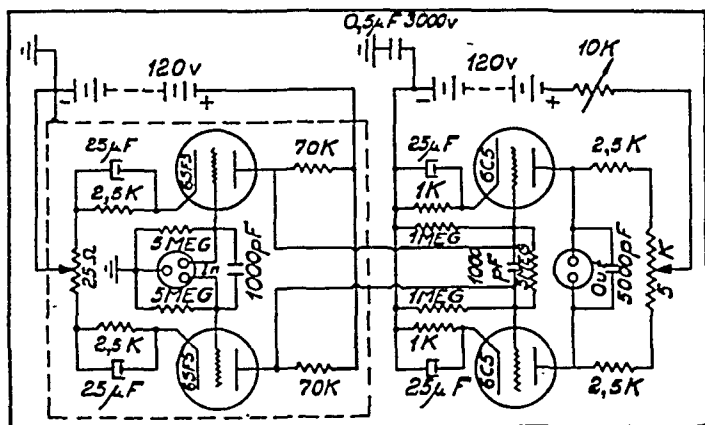


Fig. 12. Circuit diagram of amplifier of blood-pressure recorder.

The tube and pressure chamber are filled with physiol. NaCl solution, and the metal cannula itself with a solution of 5 per cent heparin (Heparin Vitrum).

The strain-gage element is made like a Wheatstone bridge with a resistance of 600 ohms. The bridge maintains its equilibrium when no pressure influences the membrane. The element is fed from a 20 V. battery, and the voltage which results when the element is brought out of balance owing to the pressure on the membrane, is recorded, after amplification, on an ordinary oscillograph (Elmqvist's principle).

The amplifier is a three-step, direct-coupled D. C. amplifier. Circuit diagram of the amplifier see fig. 12.

Owing to the severe strain caused by the detonations the element was eventually damaged, and as a new one was not available, recordings were made according to another principle in some of the later experiments. The movements of the pressure chamber membrane were then recorded by way of a metal needle direct to a microcator band supplied with a mirror. No amplification was needed in recordings of this type. In other respects the experimental devices were the same as those already described.

The blood-pressure recorder is calibrated by means of a small pressure vessel, which is connected to the copper tube instead of the cannula mentioned above. The pressure is measured with a calibrated anaeroid manometer (the same as that used in determining the blood-pressure by the bloodless method, described below).

The relation between pressure and oscillograph readings is linear.

Owing to considerable damping in the system a certain distortion of the pulse beats occurs, and the pulse curve is rounded off. As the main object in these experiments has been to measure the *average blood-pressure*, however, this distortion seems insignificant.

*The bloodless method.* For the purpose of measuring the arterial pressure in the bloodless way the above-mentioned method developed by FAHR (1938—39) and LITHANDER (1945) has been used with some slight modifications. The temperature in the thermobox has been kept at between 25 and 28° C. The

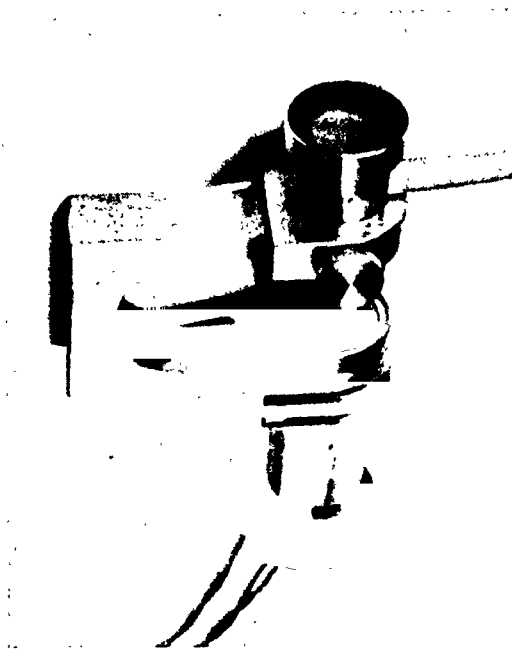


Fig. 13. Device for measuring of blood-pressure in the central artery of the rabbit's ear. The device is by means of a rubber tube, seen to the right, connected to a manometer, and placed in a thermo box not shown in the figure.

blood-pressure unit (fig. 13) consists of two adjustable brass cylinders facing each other in a metal container. The lower cylinder, which contains a small glow lamp, is covered at the top by a glass plate. The upper cylinder is closed at the bottom by a thin rubber membrane, and at the top by a magnifying glass. This cylinder is connected to an aneroid manometer by means of a rubber tube. At first a manometer was used according to von Recklinghausen-Riva Rocci and later a precision manometer (Tycos Precision Blood Pressure Meter, Taylor Instrument Companies). Both manometers have been calibrated against a mercury manometer.

The pressure has been measured within the middle part of the ear as the variations of pressure in one and the same animal are less in that part of the ear (GRANT and ROTSCHILD 1934, LITHANDER 1945). In order always to obtain a constant and shortest possible distance between the ear — always the left — and the rubber membrane, the following method has been employed: The ear is carefully placed over the glass plate of the lower tube, whereupon the upper tube is allowed to fall by its own weight against the ear. The pressure on the rubber membrane is increased to 20 mm Hg, after which the tubes are fixed in this position before the measuring begins. The systolic pressure is read off when the pulsations in the compressed vessel return, when the pressure on the membrane is again reduced.

The blood-pressure values consist of an average of 10 — in some of the earlier experiments 6 — readings, taken in quick succession.

By avoiding, as far as possible, certain errors of the method, which have

been pointed out earlier by ANDERSON, KURAYA, FAHR and LITHANDER, among others, quite good values have been obtained. The percental deviation from the mean value in a series of readings is between 5 and 10 per cent.

### Circulation time.

This has been determined by a modification of the fluorescein method introduced by KOCH (1922) at which 0.2 ml of a 20 per cent solution of fluorescein-sodium has been injected into the marginal vein of one of the ears, and the time to beginning fluorescence in conjunctiva of the opposite eye has been determined.

### Oximetry.

The relative oxygen saturation of the arterial blood has been determined by means of an oximeter according to MILLIKAN (1942). The apparatus consists, in principle, of a photo-electric colorimeter which in the bloodless way determines the difference in light absorption in the spectral region of 600—800 m $\mu$  between reduced haemoglobin and oxyhaemoglobin. This principle, which has been used by MATTHES (1935), SQUIRE (1940), GOLDIE (1942), among others, has special advantages over the method elaborated by KRAMER (1935) and used e. g. by BJURSTEDT (1946), among others, by which measuring is performed on an exposed artery.

The apparatus used by the present author is a modified and improved model of Millikan's apparatus (see LINDGREN, 1947).<sup>1</sup> It has been found quite practicable in clinics, but seems not to have been used previously in animal experiments.

Measuring was performed on a skin fold on the animal's back after the hairs had been removed with a water solution of barium or strontium sulphide. When maximal vasodilatation had been reached, usually after 10 or 15 minutes, values were obtained of degree of blood content and tissue absorption (determined by the photo-electric cell sensitive to green colour) which in the course of one experiment varied only about 5 per cent.

DRABKIN and SCHMIDT (1945) have found by determinations by direct spectrophotometry that the oxygen saturation of human arterial blood under standard conditions is 98.6 per cent, and in dog's arterial blood 98.5 per cent. In clinical experiments with oximetry the initial value of 98 per cent is generally used when the subject breathes ordinary air. The present author has found, however, the initial value 100 more convenient as the oxygen saturation of the animal injured by blast can then be given directly in per cent of the initial value. As the method gives only relative values this approximation seems justified.

### Body temperature.

This has been measured by means of ordinary rectal thermometers. The thermometer has always been inserted in rectum to exactly the same length

<sup>1</sup> The apparatus has been made by K. G. Berg, Civ. Eng. Stockholm.

in all readings, and kept in this position by an assistant. This seems important according to, for instance, TACHAU (1912), SAATHOFF (1914), ANDERSEN (1943) and WALLDÉN (1944) who in their clinical investigations have shown that the temperature is higher further up in rectum. It is also important that the animal is calm during the entire reading, as otherwise the temperature may rise several tenths of a degree.

All rectal thermometers used here have been calibrated against a standard thermometer.

### C. Biochemical methods.

Biochemical investigations of changes in some of the chemical components of the blood have been made in a number of animals which were allowed to live for some time after the detonation.

After incision in the marginal vein of the ear, blood has been tapped into a graduated suction tube in which a moderate vacuum has been obtained by means of a suction pump. The suction tube has been specially constructed for the purpose by the present author.

For determination of non protein nitrogen, creatinine, phosphorus and potassium, samples of blood have been tapped into bottles containing sodium oxalate gauze. For all other samples 1 drop of 5 per cent heparin for every 5 ml of blood has been used, to prevent coagulation.

Double determinations have been made as a rule. The error of method has been determined for all methods used. This has been calculated from a number of double determinations (see page 40). The methods are as follows:

#### Haemoglobin.

This has been determined as reduced haemoglobin after haemolysis with a solution of 0.1 per cent sodium carbonate and reduction with sodium ditionite, in a Pulfrich photometer. The factor 16.1 given by HEILMEYER and MUTIUS (1938) has been used. This is possibly somewhat high, but as it is only the relative changes in the haemoglobin content which are of interest here, this seems unimportant. The haemoglobin values are given in g Hb/100 ml of blood. 51 double determinations gave an average difference of  $0.026 \pm 0.023$  g Hb/100 ml with an error of the method of 0.17 g Hb/100 ml, or a percental error of 1.6% of the mean value in single determinations, and an error of 0.12 g Hb/100 ml or 1.1% of the mean value in double determinations.

#### Haematocrite.

The volume of the blood cells has been determined in an ordinary angle centrifuge by using haematocrite tubes according to VAN ALLEN (1925). The tubes have been turned for 30 minutes with 3500 revolutions per minute. 50 double determinations showed a mean difference of  $0.24 \pm 0.21$  % with an error of the method of 1.5% haematocrite or 4.0% of the mean value in single determinations, and an error of 1.0% haematocrite or 2.8% of the mean value in double determinations.

### Blood sugar.

This has been determined according to HAGEDORN, HALSTRÖM and JENSEN (1935). From 50 double determinations a mean difference of  $2.3 \pm 1.0$  mg/100 ml was obtained with an error of the method of 5.5% in single determinations, and 3.9% in double determinations.

### Plasma chlorides.

The chloride content of plasma has been determined by the electrometrical method according to a modification of Müller's method elaborated by NORBERG (1949). All values are given in m mol.

The mean difference of 39 double determinations was  $0.26 \pm 0.41$  m mol. The error of the method: 2.6 m mol or 2.6% of the mean value and 1.8 m mol or 1.9% of the mean value in single and double determinations, respectively.

### Alkali reserve.

The carbon dioxide binding capacity of plasma has been determined by the diffusion method according to CONWAY (1947). Values are given in volume per cent. The mean difference of 42 double determinations was  $0.43 \pm 0.55$  vol %. The error of the method: 3.6 vol % or 6.8% of the mean value in single determinations, and 2.5 vol % or 4.8 % of the mean value in double determinations.

### Plasma proteins.

Total protein in plasma has been determined by a diffusion method mainly according to CONWAY (1947). 50 double determinations showed a mean difference of  $0.06 \pm 0.06$  g/100 ml. The error: 0.39 g/100 ml or 6.2% of the mean value and 0.28 g/100 ml or 4.4% in single and double determinations, respectively.

### Non protein nitrogen and creatinine.

Non protein nitrogen and creatinine have been determined according to FOLIN.

### Phosphate.

The content of acid soluble phosphorus in plasma has been determined by a method according to FISKE and SUBBAROW and modified by TEORELL (1931). The mean difference of 49 double determinations was  $0.0004 \pm 0.006$  mg/100 ml. The error: 0.04 mg/100 ml or 1.1% of the mean value in single determinations, and 0.03 mg/100 ml or 0.8% in double determinations.

## Potassium.

After dry incineration of plasma at 500° C and precipitation as chloroplatinate the potassium has been determined by a modification, adapted for photometry, of a method elaborated by NORBERG (1938). A Klett-Summerson photometer has been used. The mean difference of 51 double determinations was  $0.026 \pm 0.045$  m mol. The error: 0.32 m mol or 6.8% of the mean value in single determinations, and 0.23 m mol or 4.8% in double determinations.

## Carbon monoxide.

Carbon monoxide in blood has been determined by a method described by WENNESLAND (1940). The values are given in vol %.

49 double determinations gave a mean difference of  $0.016 \pm 0.04$  vol %. The error was 0.28 vol % or 7.6% of the mean value in single determinations, and 0.20 vol % or 5.4% in double determinations.

## Iron.

In addition to the method described on page 53 below for determining the extent of lung haemorrhages, this has also been done, in a number of cases, by determining the iron content in the lung and in samples of blood from the same animal. These determinations have been made according to the method introduced by BREUER and MILITZER (1938) and which has been modified by the present author:

### a. Determination of iron in lung.

The lung which has been dried to constant weight in a 250 ml flask of pyrex glass at a temperature of 110° C, is incinerated on a sand bath (270° C) with 10 ml of conc. sulphuric acid (pro analysi). The solution clarified by an addition of perhydrol, is allowed to cool, after which it is diluted in a measuring flask to exactly 250 ml. 10 ml of this solution is mixed with 0.5 ml of a saturated solution of potassium peroxydisulphate (10 g  $K_2S_2O_8$  in 100 ml aq. dest.) and 2 ml of a 3 N solution of potassium rodanide (146 g KCNS + 20 ml of acetone purum diluted with aq. dest. to 500 ml). Photometry against a reagent blank should be done as soon as possible. A photometer with blue filter (BG 7, wave-length 475 m $\mu$ ) according to LANGE, has been used.

The iron content is obtained from a standard curve. As standard a solution of Mohr's salt has been used containing 1 mg Fe per ml ( $1.757$  g  $FeSO_4 \cdot (H_4N)_2SO_4 \cdot 6 H_2O$  dissolved in 250 ml N  $H_2SO_4$ ).

Double determinations have been made. The mean difference of 23 double determinations was  $0.0043 \pm 0.0055$  mg Fe. The error of the method: 0.03 mg Fe, making 2.3% of the mean value in single determinations, and 0.02 mg Fe or 1.6% in double determinations.

## b. Determination of iron in blood.

0.5 ml of blood is incinerated in a 50 ml pyrex flask with 2 ml of conc. sulphuric acid on a sand bath at 270° C. Perhydrol is added till a clear solution is obtained. When incineration is completed the sample should not be dark-coloured one hour after the final admixture of perhydrol. It is allowed to cool and is then diluted with aq. dest. to exactly 50 ml. From this solution a 10 ml sample is taken which is treated with  $K_2S_2O_8$  and KCNS, and read off as above. The values are given in mg Fe/ml of blood. With the help of this value and the value of the iron content of the lung we can then determine the amount of blood in the lung. In doing that the content of tissue-bound iron in the lung has been left out of account as this would be unimportant compared to the iron content of the blood.

The mean difference of 24 double determinations is  $0.0009 \pm 0.001$  mg/ml. The error of the method has been estimated at 0.007 mg/ml or 2.2% of the mean value, and 0.005 mg/ml or 1.6% in single and double determinations, respectively.

## D. Statistical methods.

The arithmetical mean  $M$  with its standard error  $\epsilon(M)$  and the standard deviation  $\sigma$  have been calculated according to current formulae. The significance of the differences between different groups of observations have been estimated by analysis of variance and the  $t$ -test in accordance with formulae given by FISHER (1936) and BONNIER and TEDIN (1940).

From the variance quotient and the figure  $t$

$$t = \frac{M_1 - M_2}{\sqrt{\epsilon(M_1)^2 + \epsilon(M_2)^2}}$$

the probability  $P$  has been obtained which states whether a significant difference exists or not between different materials.

$P > 0.05$ , states the difference as not significant i. e. that the materials belong to the same population.

$0.05 > P > 0.01$  states the difference as probable.

$0.01 > P > 0.001$  states the difference as highly probable.

$0.001 > P$  states the difference as statistically significant.

The error of the methods have been calculated from a number of double determinations. The mean value  $d$  of the differences and their standard deviation  $\sigma_d$  has been computed. If  $d$  is not significant i. e.  $d < 3 \sigma_d$ , the standard deviation of a single observation is calculated according to the following formula:

$$\sigma_s = \frac{\sigma_d}{\sqrt{2}}$$

The error of method is then  $\sigma_s$  in single observations, and  $\frac{\sigma_s}{\sqrt{2}}$  in double observations.

The correlation co-efficient  $r$  which states the strength of the correlation between two variables  $x$  and  $y$ , measured in pairs, has been calculated according to the following formula (see WORTHING and GEFFNER, 1944):

$$r = \frac{n \sum xy - \sum x \cdot \sum y}{\sqrt{\{n \sum x^2 - (\sum x)^2\} \{n \sum y^2 - (\sum y)^2\}}}$$

The correlation is considered strong if  $r > 0.5$  (see e.g. ESSEN-MÖLLER 1941).

The mean error of the correlation coefficient  $\varepsilon(r)$  has been calculated in accordance with the following formula:

$$\varepsilon(r) = \frac{1 - r^2}{\sqrt{n}}$$

which gives sufficient degree of accuracy provided  $r$  is not quite equal to 1 or 0 (WORTHING and GEFFNER).

In the tables below  $n$  always indicates the number of animals or experiments.

## CHAPTER 6.

### Detonation Chamber with Impulse Pendulum.

In field blastings it is easy to obtain high *maximum pressures* variable at will by increasing the size of the charge and reducing the distance from it. The *impulse*, on the other hand, cannot be increased to a similar extent, and very great impulses at relatively moderate maximum pressures — for instance such pressure and impulse conditions which occur when an atomic bomb detonates — cannot be obtained in this way. The greatest impulses are obtained from detonations in confined spaces where the pressure increase lasts for a considerably longer time than in detonations in the field.

As it seemed desirable also to study the effect on the organism of blast waves with great impulse a detonation chamber has been constructed specially for animal experiments.<sup>1</sup> Apart from the very high impulse values which can be obtained by using it the detonation chamber also offers several other advantages. For instance, it is possible to obtain with only a few grammes of explosive in the detonation chamber, pressure and impulse values similar to those we get in the field when hundreds of kg of explosives are used. Furthermore, it is possible to use physiological recording instruments which

<sup>1</sup> The detonation chamber has been constructed in collaboration with E. von Zeipel, Ph. L.



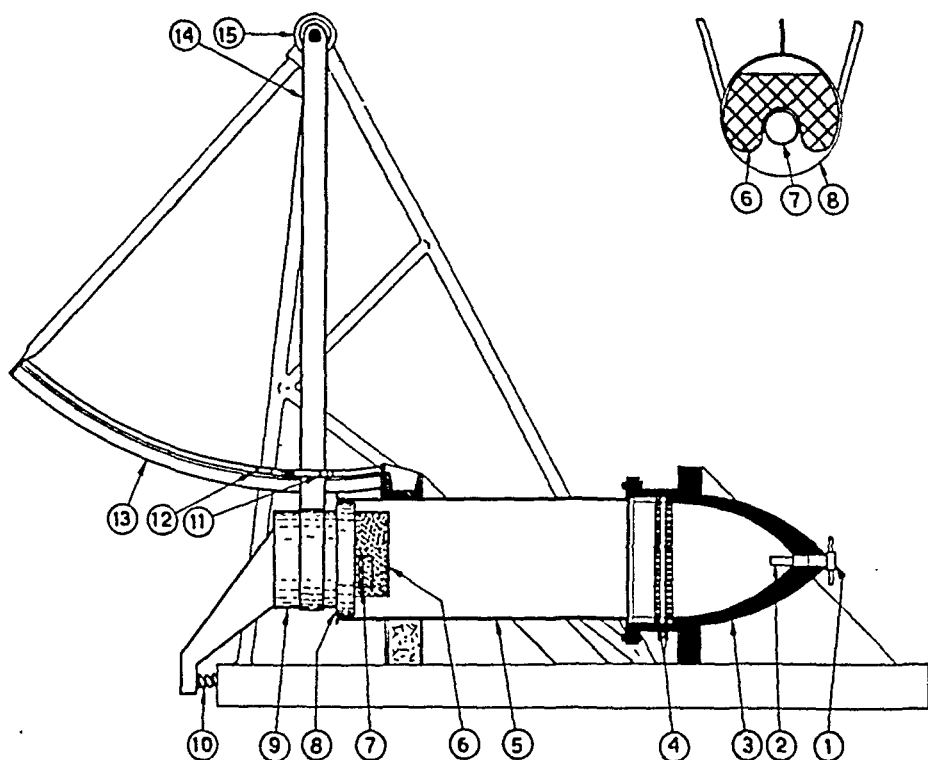


Fig. 14. Plan of the detonation chamber. Scale 1 : 15. The figures correspond to those in the text.

cannot be used in field blastings owing to their sensibility to outside disturbing influences, such as variations of pressure.

The detonation chamber which is shown in figs. 14 and 15, consists mainly of two parts, namely, the detonation chamber itself (3) with an extension tube (5), and a pendulum for impulse determination (8, 9, 14) on which the animal cage and a maximum pressure gauge (7) are placed.

The detonation chamber is an iron cone of relatively thick construction (3) — it is made of the top of a 500 kg bomb — which has been lengthened by an iron tube (5) fastened to it with screws. The length of the chamber including the extension tube is 122 cm, and its inside diameter is 40 cm. In the top of the iron cone is a threaded screw plug (1) where the electric blasting cap is inserted. When the screw plug is in place the charge, which is fastened to the detonator, will be inside the cone and approximately 10 cm from its top.

Where the conical part of the chamber meets the extension tube two iron sheets (4) have been placed at a distance of 2 cm from each

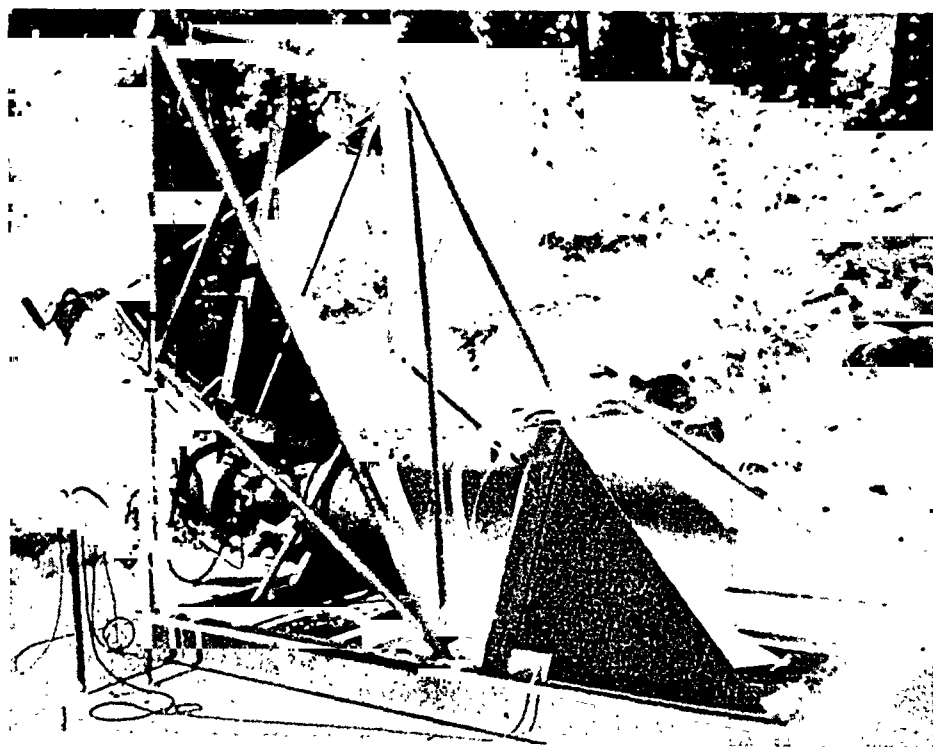


Fig. 15. The detonation chamber.

other. They are perforated, the holes being 10 mm in diameter and at a distance of 10 mm from each other. The sheets completely cover the cross-section of the chamber and can be moved parallelly with each other so that the holes of one sheet are covered more or less by the other sheet, thus completely or partly, screening off the extension tube from the detonation chamber itself. By this device the impulse can be varied within quite broad limits. The sheets are removable and have only been used in blast experiments when small pressures and very great impulses have been aimed at.

Before the detonation the open end of the detonation chamber is closed by means of a wooden disc (8). This is fastened on the body (9) of the impulse pendulum and fits the inside diameter of the chamber with very little allowance. On the inside of the wooden disc are mounted, as mentioned before, a maximum pressure gauge (7) and a cage for the animal (6). Before the impulse from the detonation has caused the pendulum to swing out the animal and pressure gauge are, therefore, inside the chamber at a distance of approximately 100 cm from the charge.

The impulse pendulum is suspended on ball-bearings (15) in a tubular steel stand. The body of the pendulum (9) consists of a cylindrical leaden weight of 190 kg. The frontal area of the pendulum, i. e. the wooden disc, is 1250 cm<sup>2</sup>. The length of the pendulum measured from the centre of the suspension axis to the centre of the pendulum body, which coincides fairly well with the centre of gravity of the pendulum, is 180 cm. The total weight of the pendulum, pressure gauge and animal cage is about 230 kg.

When the explosion causes the pendulum to swing out it brings with it a small slider (12) which glides along a peripheral rail. The slider is stopped in the pendulum's dead centre owing to some small friction, and the angular deflection of the pendulum is registered.

The impulse received by the pendulum is obtained from the formula of the impulse pendulum, given above. Because of the great mass of the pendulum the friction term  $\frac{2K\varphi}{m}$  may be disregarded here. The chord between the starting and finishing position of the slider on the rail is proportional to the factor  $\sqrt{1 - \cos \varphi}$ , and the impulse, therefore, is proportional to the length of the chord. Thus this length multiplied by the constant 0.54, which is calculated from the formula, gives the impulse in gf. sec/cm<sup>2</sup>.



Fig. 16. The detonation chamber. The pendulum body with the animal cage.

After the detonation the pendulum does not swing back towards the opening of the detonation chamber but is arrested by a couple of blocking pieces which have been pushed by the pendulum towards a strong brake rim (13) below the rail. In this way the pendulum is arrested almost immediately after it has turned. If this brake appliance, for some reason or other, should stop to function there is also a safety device consisting of a strong coil spring (10) which stops the movement of the pendulum just before it has reached zero. In case of faults in the brake arrangement this device will then prevent the instruments mounted on the wooden disc from being smashed to pieces.

The pressure gauge, which is fastened to the centre of the 60 mm thick circular wooden disc of the pendulum body, is of the same construction as those used in the field blastings. On the disc hangs also the cage (see fig. 16) in which the animal is placed. The envelope surface of the cage is made of perforated sheet metal, the side facing the chamber is of coarse-meshed wire netting, and the side touching the wooden disc is of iron plate. The cage is secured to the

disc by means of hooks and can be removed quite easily. In the cage and disc there are holes for the cables of the different instruments.

In the experiments carried out in the detonation chamber a plastic explosive has been used as charge. Weights of charge of 1 to 6 g have been used.

## CHAPTER 7.

### The Errors of the Pressure and Impulse Determinations.

In stating the physical effect of the shock wave on the animals, maximum pressure and impulse values have been determined for each rabbit in the experiments. *In experiments with "group type A" these values have been obtained from the pressure and impulse gauge closest to the animal in question. In experiments where several recordings have been taken at different distances from the charge ("group type B"), on the other hand, the pressure and impulse values have been calculated from diagrams of pressure-distance and impulse-distance, determined for each weight of charge used.*

The values for each particular animal are, of course, impaired by a certain amount of error. This is, first and foremost, composed of the possible systematical and random errors by which the recording apparatus itself is impaired, and also of the error resulting from the fact that the pressure and impulse field around a detonating charge deviates in varying degrees from spherical symmetry (below called the azimuthal dispersion of the pressure and impulse values).

#### The error of the maximum pressure determination.

As emphasized previously the maximum pressure, which acts on a large, flat wall, is quite a different pressure compared to that which acts on a very small object. Furthermore the pressure determined by the pressure gauges used in these experiments is yet another. This is because the pressure received by an object depends, to a large extent, on the physical qualities of this object, for instance, its shape, density, the nature of its surface, etc. The pressure, which

is given here as the maximum pressure acting on an animal, is actually the pressure which has acted on the membrane of the pressure gauge closest to this animal. It is, therefore, dependent on the changes in the shock wave brought about by its being dammed up, reflected and deflected against the gauge. These changes, which are rather hard to determine quantitatively, mainly depend on the appearance of the shock front and on the external shape of the pressure gauge. It is obvious, therefore, that a certain error will be admitted when the pressure obtained from a pressure gauge is assigned to the animal closest to it. However, as both the form of the shock wave and the shapes of the pressure gauges and of the animals have been mainly constant in the different experiments, this systematical error should justly be the same in all experiments, and can, therefore, be disregarded. When comparing the pressure values obtained in this study with those given earlier in the literature on this subject, on the other hand, it must unavoidably be taken into consideration.

In comparative investigations (VON ZEIPPEL 1946), between the mechanical maximum pressure gauges used here and piezo-electric gauges with crystals of Rochelle salt, quite considerable systematical differences have been found between the values obtained. This evidently depends on the fact that the mechanical gauges indicate the total of static and dynamic pressure, while the piezo-electric gauges, for the main part, give only the static pressure. The latter gauges, therefore, are little dependent on the orientation in the pressure field, while, on the other hand, the former give quite different values if orientated parallelly with or perpendicularly to the incident shock wave. Furthermore, the membrane of the mechanical gauge owing to slight damping, makes a deflection which is approximately 100 per cent too great. All the pressure values, excepting those obtained in the detonation chamber, have been corrected of this error. This was done by multiplying the recorded deflection of the membrane with a reducing factor  $K$ , which has been determined through experimental investigations and theoretical calculations based on a general knowledge of the nature of the shock wave and the physical qualities of the gauge membrane.

The pressure values obtained in the detonation chamber have not been corrected as the excess deflection of the gauge membrane would be quite insignificant here, owing to the considerably slower progress of the detonation in the chamber (see fig. 24, page 93).

The reducing factor  $K$  differs for gauges with different thicknesses of membrane, and varies in thin membranes with the size of the charge.

In the experiments with "group type A," where small charges up to approximately 1 kg have been used,  $K_2^1$  is  $0.57 \pm 0.02$  (the error is estimated).

In the experiments with "group type B" and bigger charges, the following values have been used with regard to  $K$ :

Weight of charge in kg	$K_1$	$K_2$	$K_4$
1.25—5	$0.36 \pm 0.06$	$0.47 \pm 0.03$	$0.52 \pm 0.01$
> 5	$0.24 \pm 0.06$	$0.47 \pm 0.03$	$0.52 \pm 0.01$

These  $K$  values have been determined as mean values from a number of recordings made at several detonations each with its own weight of charge. The errors are estimated. Owing to difficulties of reading off, the error of the value increases, if the deflections of the membrane are very small.

The error, which is about 4 per cent in the recordings usually obtained at a few hundred  $\mu$ , can, for the main part, be estimated to depend on the uncertainty of the reducing factor  $K$ . It increases rapidly if the deflection is  $< 10 \mu$ .

**The error of the pressure values owing to deviation of the shock wave from spherical symmetry.**

The greatest error of the maximum pressure, particularly when only one or two pressure gauges have been used at each blasting, is most likely due to the azimuthal dispersion of the pressure field. The fact is that owing to the shape and inhomogeneity of the charge the pressure will not be exactly the same in different points of recording at the same distance from the charge. Consequently, the maximum pressure recorded with a certain pressure gauge can, only within certain very strict limits, therefore, be attributed to the animal closest to it, without an even greater error being attached to the value. The greater the angular distance is between pressure gauge and animal the greater is this error.

<sup>1</sup> The index of  $K$  states the thickness of the membrane in mm.

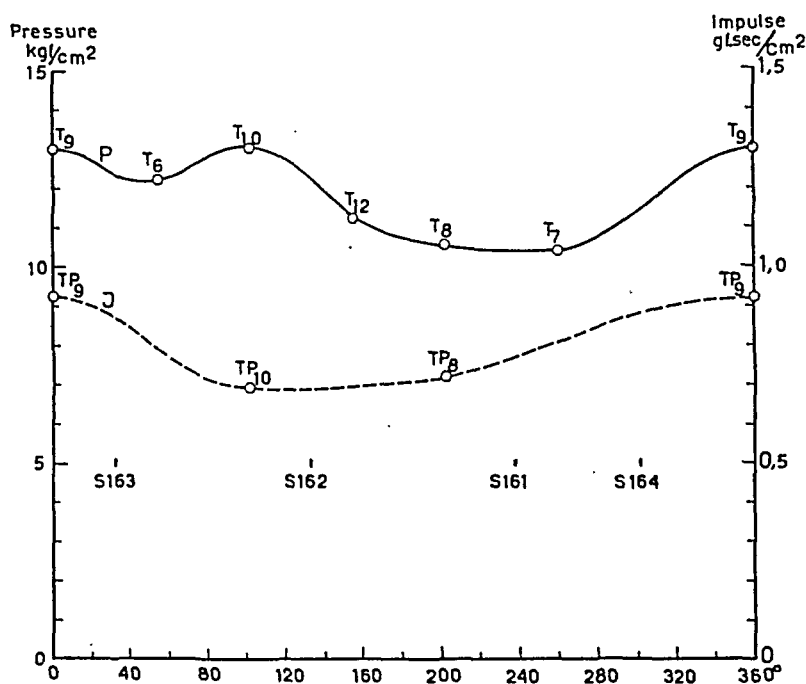


Fig. 17. The dispersion of the pressure and impulse values (azimuthal dispersion) in a blasting experiment with group type A. — = max. pressure and - - - = impulse. Connecting lines drawn by hand. T and TP: see fig. 4, page 24. S 161—S 164 indicate the places of four rabbits.

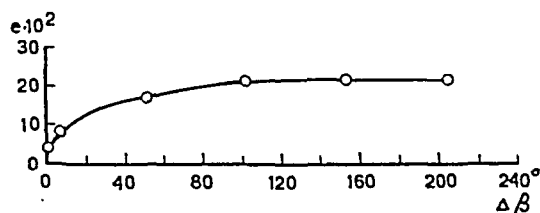


Fig. 18. The uncertainty of the max. pressure value acting on an animal as a function of the angular distance ( $\Delta\beta$ ) from the animal to the pressure gauge.  $e$  is the relative uncertainty, and thus the diagram gives the uncertainty in per cent.

In order to determine the magnitude of this error, 6 pressure gauges in each of 6 experiments (blast experiments nr 64—69) have been grouped around the charge at exactly the same distance from this ("group type A"). The pressure and impulse values obtained and their azimuthal dispersion in one of these experiments, is shown in fig. 17.

The relative error of a maximum pressure ascribed to a rabbit was shown in these experiments to depend on the angular distance



between the animal and the pressure gauge. This relation is shown in fig. 18.

*In experiments according to "group type A" the probable error increases rapidly to  $\pm 10$  per cent when the angular distance between pressure gauge and animal increases from 0 to  $10^\circ$ , and it rises to  $\pm 20$  per cent at  $100^\circ$  to decrease again when the angular distance reaches  $260^\circ$ .*

In experiments according to "group type B" the maximum angular distances between the different pressure gauges were approximately  $18^\circ$ , and the maximum angular distance between an animal and the gauge closest to it, was  $\pm 3^\circ$ . For that reason the error due to azimuthal dispersion may be omitted. The pressures acting on the rabbits have been calculated by interpolation in a pressure-distance diagram determined for each experiment. To estimate the error of these pressure values, is very difficult. *With regard to the distribution of the pressure values along a smoothing curve the error in determining the pressure for one particular animal could reasonably be expected to be  $\pm 10$  per cent in experiments of "group type B."*

### **The error of the impulse determination.**

Concerning the error of the impulse values ascribed to one particular animal practically the same applies as in regard to the error of the pressure values.

The impulse gauges used here (impulse pendulums and "drop cylinders") state with sufficient accuracy the impulse acting on them, but cannot give an exact account of the impulse received by, for instance, a big flat wall, or by an animal (rabbit). Owing to the uniformity of the experiments, however, this systematical error has been disregarded here, as in the max. pressure determinations, and the impulse has been ascribed to the animal closest to the gauge.

A comparison between the impulse values obtained with the impulse pendulum and those obtained with the "drop cylinder" showed that in the experiments with "group type A" the values obtained with the impulse pendulum was throughout the experiments about  $0.1 \text{ gf} \cdot \text{sec/cm}^2$  less than the impulse values obtained with the "drop cylinder." This difference appears to be due to movements caused by the detonation in the stand supporting the pendulum, which thus affected the deflection of the pendulum. As the impulse values obtained with the pendulum have a greater error (see below)

than those obtained with the "drop cylinder" the values in all experiments with "group type A" have been corrected to "drop cylinder" values, that is to say, the values obtained with the pendulum have been increased by  $0.1 \text{ gf} \cdot \text{sec}/\text{cm}^2$ .

*The error of a single pendulum impulse value* is dependent on the angular deflection of the pendulum ( $\varphi$ ). Thus it is about 4 per cent for  $\varphi > 20^\circ$ . It increases rapidly when  $\varphi$  falls below  $5^\circ$  and amounts to about 22 per cent when  $\varphi$  is  $1^\circ$ .

*The error of a single "drop cylinder" impulse value* appears mainly to be dependent on the error ( $dl$ ) in measuring the throwing-distance.  $dl$  was usually approximately 1 cm in these experiments. Only at very great throwing-distances, i. e. at high speed of the "drop cylinder" resulting in ill-defined point of landing,  $dl$  rose to 5 cm or more.

The error of a single "drop cylinder" impulse value is almost always below 3 per cent, and generally about 1 per cent.

In experiments with "group type B" the impulse has not been measured with the "drop cylinder" due to technical reasons. In these experiments the impulse values obtained with the pendulum have not been corrected as above.

#### **The error of the impulse values owing to deviation of the shock wave from spherical symmetry.**

As to the error of the impulse value in consequence of the azimuthal dispersion the same consideration applies as regards the maximum pressure values. In discussing the error as a function of the angular distance between pressure gauge and animal, this was found to be between 10 and 20 per cent. This applies to the impulse also, *and the impulse values in experiments with "group type A," therefore, are given with an error of  $\pm 15$  per cent.*

In experiments of "group type B" the azimuthal dispersion error with regard to the pressure values, was unimportant in comparison to influences from the stand etc. *Analogous to the pressure values the impulse received by one particular animal was estimated as having an error of  $\pm 10$  per cent.*

## CHAPTER 8.

**Objective Determination of the Lung Blast Injury.**

Several authors have pointed out that the extent of lung injury is closely correlated to the violence of the detonation. The present author has also found in a large number of experiments with over 600 animals and under greatly varying external conditions, that the lung injuries can be used as a kind of "indicator" of the destructive capacity of the blast wave.

Already at quite an early stage it was clear that a subjective estimate only of the extent of the lung injury was not sufficient, as extremely uncertain results were obtained even if the estimation was carried out by one and the same person. It was obvious, therefore, that if the lung injury should be used as a measure of the degree of blast injury, it must be recorded by an objective method.

In earlier experiments where the blood content in the lung or other organs has been determined, the *intravasal* blood quantity has been determined in most of the cases.

MENICANTI (1894) soaked the blood from the sectioned lungs with water, after which he determined the blood quantity colorimetrically with blood from the heart as comparative solution. A similar method was used by BRUNS (1909, 1912). He tied the lung vessels and soaked the haemoglobin with 0.1 N hydrochloric acid (1909) or aq. dest. (1912), and determined it with blood from vena cava as comparison.

SJÖSTRAND (1934) and HANSSON and SJÖSTRAND (1934) determined the capillary blood quantity in microscopic slides of lungs where the corpuscles had been coloured with orto-toluidine. The water content was determined by drying to constant weight.

Other methods used in determining the blood content in organs are, for instance, determination of the iron content, of volume changes of the organ by means of a pletysmograph, determination of in- and out-flowing blood with the aid of a stromuhr, etc. (see SJÖSTRAND 1934).

In all these experiments the only object has been to determine the *intravasal* blood content of the lung.

A method for determining the size of *lung haemorrhages* in cats exposed to strong deceleration has recently been elaborated by McDONALD and KELLEY (1947). The lungs were perfused *in situ*, with vena cava inf. and sup. ligated, with a salt solution until the lung circulation was completely free from blood, after which the lungs were dried, pulverized and freed from the remaining haemoglobin which was determined as pyridin-haemochromogen according to FLINK and WATSON (1942).

None of these methods, however, seemed sufficiently simple to be used in serial experiments. The present author, therefore, has used a modification of a method elaborated by BOREI (1944), whereby the weight increase of the lung, caused by haemorrhage, is determined in relation to the calculated normal weight of the same lung. That quite a large increase in weight may occur has been shown by COHEN and BISKIND (1946) in autopsies, and in animal experiments by CLARK and WARD (1943) and SHELLY and HORWATH (1946), who state, that lung weights up to 4 and 5 times the normal weight have been found in animals injured by blast.

The weight increase of the lung injured by blast, which may be due to haemorrhage as well as to oedema fluid, is given here through a quotient (from now on called quotient of haemorrhage):  $\frac{Vi_0}{Vi_1}$ , where  $Vi_1$  = weight of lung injured by blast,

and  $Vi_0$  = calculated normal weight of the same lung.

$Vi_0$  can be determined if we know the body weight of the animal and the weight of the rabbit's lung in per cent of the body weight.

*Methodics:* The lungs are removed separately and bronchi and vessels cut away close to hilus, after which the lungs are washed quickly in water. The blood in the larger vessels is squeezed out, and the lungs are dried by lightly pressing them between filter paper, and weighed. Animals, which have not been anaesthetized during the experiment, have been given 1.5—2 ml of Narcotal immediately before being killed, as it has been found that more constant values for lung weight in per cent of body weight are obtained in this way, than if no anaesthetic has been given. In the first case the values are even somewhat lower, which possibly could be explained by the fact, that the quantity of blood deposited in the lung may be smaller in anaesthetized animals (SJÖSTRAND 1934, HANSON and SJÖSTRAND 1934). By this method the blood in the large vessels would be almost entirely drained off, while blood in the small vessels and capillaries and extravasal blood would not be influenced to any large extent.

Pressing the lung between filter paper, of course, introduces a certain degree of error in the determination in spite of the fact that it has always been carried out in as similar a manner as possible in each experiment. The magnitude of this error has been determined as follows: The lungs have been prepared and weighed, as described

above. Then they have been pressed again more thoroughly between new filter papers. These control experiments include 118 lungs from 59 animals. By the renewed and more thorough pressing we get a reduction of the weight of the lung amounting to an average of  $2.2 \pm 0.2$  per cent ( $\sigma = 1.6$ ) for left lung, and  $2.3 \pm 0.2$  per cent ( $\sigma = 1.9$ ) for right lung.

#### Determination of lung weight in per cent of body weight.

This determination is based on the assumption that the weight of the lung is correlated to the weight of the body within quite narrow limits. This relation has been determined both in animals from the same stock as those used in the blast experiments, and also in 9 animals from quite a different stock. The results are found in tab. 1.

TABLE 1. Weight of rabbit lung in per cent of body weight and the correlation coefficient  $r$  of the correlation between lung weight and body weight.

Animal	Number of animals	Lung weight in per cent of body weight		Correlation coefficient $r \pm \varepsilon(r)$	
		Left lung	Right lung	Left lung	Right lung
Rabbits of the same stock as the experimental animals used..	49	$0.16 \pm 0.02$	$0.23 \pm 0.03$	$0.84 \pm 0.04$	$0.81 \pm 0.05$
Rabbits of quite another stock .....	9	$0.15 \pm 0.01$	$0.22 \pm 0.02$	$0.99 \pm 0.01$	$0.92 \pm 0.03$

The correlation between lung weight and body weight for these two groups of animals has also been determined mathematically, and the correlation coefficient  $r$  and its standard error  $\varepsilon(r)$  have been determined. The values obtained are also found in tab. 1.

The correlation is apparently very strong. The agreement between the values from the two different groups of animals is also very good.

The values also correspond with those given for rabbit lungs by HANSSON and SJÖSTRAND (1934), namely,  $0.16 \pm 0.0064$  and  $0.22 \pm 0.0065$  for left and right lung, respectively.

The values given in tab. 1 apply to rabbits with a body weight of between 1 and 3 kg.

The dry weight of the lung also varies within quite narrow limits in relation to the body weight, and determination of the dry weight of the lungs — dried to constant weight at 120° C — has been used for the purpose of control in a number of cases. The dry weight of the lung in per cent of the body weight and the correlation coefficients for this relation are found in table 2.

TABLE 2. Dry weight of rabbit lung in per cent of body weight and the correlation coefficient  $r$  of the correlation between these two variables.

Animal	Number of animals	Dry weight of the lung in per cent of body weight		Correlation coefficient $r \pm \varepsilon(r)$	
		Left lung	Right lung	Left lung	Right lung
Rabbits of the same stock as the experimental animals used..	26	$0.028 \pm 0.003$	$0.041 \pm 0.004$	$0.93 \pm 0.03$	$0.82 \pm 0.06$
Rabbits of quite another stock .....	9	$0.028 \pm 0.003$	$0.040 \pm 0.003$	$0.82 \pm 0.12$ ( $n = 8$ )	$0.82 \pm 0.11$

*The total error of the method for determination of lung haemorrhages is about 13 per cent.*

#### Determination of volume increase of the blasted lung.

A more or less pronounced increase of the lung volume is seen in blasted animals. The lung becomes the seat of a traumatic emphysema and fails to collapse in a normal way when the thoracic cavity is opened.

To obtain an objective measurement of the degree of volume increase a method, similar to that for stating the weight increase, has been used. The volume increase, then, is given by the following quotient:

$$\frac{Vol_1 - Vol_{bl}}{Vol_0}, \text{ where } Vol_1 = \text{volume of lung injured by blast,}$$

$Vol_0$  = calculated normal volume of the same lung,  
 $Vol_{bl}$  = volume of extravasated blood.

The volume increase of the lung injured by blast is partly, or perhaps mainly, composed of the emphysematic inflation of the lung but also to a certain extent, of course, of the haemorrhages. The latter component's share in the actual volume increase, however, is very hard to determine with any accuracy whatsoever, as the relation between inter- and intra-alveolar localization of

haemorrhage varies considerably from case to case. For that reason it has been found more convenient to exclude altogether the share conditioned by the haemorrhage (+oedema).

$Vol_1$  is obtained by determining the displacement of the lung, by immersing it in water by means of a small lead weight.  $Vol_0$  is obtained from the weight of the blood ( $V_{i_1} - V_{i_0}$ ) and its specific gravity. The specific gravity of rabbit blood has been determined pycnometrically on 15 samples of blood, and was  $1.045 \pm 0.00095$  ( $\sigma = 0.004$ ).  $Vol_0$  for the lung of a particular animal has been calculated from the specific gravity of the normal rabbit lung and the calculated normal weight of the lung. The specific gravity has been determined in a number of animals, and the values obtained are found in table 3.

TABLE 3. Specific gravity of rabbit lung.

Animal	Number of animals	Specific gravity of the lung	
		Left lung	Right lung
Rabbits of the same stock as the experimental animals used.....	49	$0.73 \pm 0.06$	$0.72 \pm 0.05$
Rabbits of quite another stock.....	9	$0.72 \pm 0.08$	$0.74 \pm 0.03$

## CHAPTER 9.

### The Development and Localization of Blast Injuries.

#### The development of blast injury.

After the detonation it has been possible in most of the cases to examine the animals within one minute. Only in blastings with the biggest charges this time has been somewhat extended, owing to the safety distance from the charge, but in no case has it ever exceeded 5 minutes.

Already at the first inspection the animals, especially the most severely injured, show a marked apathy. When they have been taken out of their cages they are unwilling to jump and move only with the utmost unwillingness. If they are made to move this is done with a slow shuffling gait. The severely injured animals often show difficulties in sitting and preferably lie down on their sides with their legs stretched out, while the head is raised forward and up, to make breathing easier.

Already within a minute after the detonation a pronounced dyspnoea is seen in many of the severely injured animals, which, within the next 10 to 30 minutes, often increases to a violent air hunger with vigorous breathing and maximum use of the auxiliary respiratory muscles. A more or less severe cyanosis often sets in. It is usually more pronounced in unanaesthetized animals.

The animals react only slowly to impressions of light, and often not at all to impressions of sound, the latter probably owing to injured ear drums. Often a transient miosis is noticed.

The severely injured animals do not feed nor drink, while the less injured animals often feed immediately after the detonation, if given food. Spontaneous passing of urine and faeces occurs in severely injured animals.

The changes of body temperature after the detonation will be discussed in chapter 15.

After external examination the animals not used for physiological studies, have been killed. They have been anaesthetized with 1.5- 2 ml of Narcotal. From this dose, given in quick intravenous injection, they generally die within a short time without cramps. This is important, as it has been found that animals, which have had cramps before dying, often show haemorrhages in the lungs. When respiration has ceased the blood is tapped from the cut carotid arteries. Before this is done it has always been made sure, that respiration has actually ceased, thus preventing the animal from aspirating blood down into the lungs should trachea have been injured when the carotid arteries were cut.

The time interval between the detonation and when the animal was killed, except for animals on which physiological observations have been made, has been kept constant, as far as possible. It has usually been between 10 and 15 minutes. This seems fairly important in view of the fact that the pulmonary haemorrhages caused by the detonation are not stationary as bleeding may continue for quite a long time. Thus HADFIELD (1941) states that the haemorrhages have reached maximum degree after approximately 4 hours, and ROBERTS (1940) and HADFIELD and CHRISTIE (1941) are of opinion, that haemorrhages from the capillaries may continue for several days.



## Localization of lesions in blast injury.

### A. External injury.

One of the characteristic features of the blast syndrome is the existence of more or less severe injuries to internal organs, while external lesions are very rare. Only when the detonation is of extreme violence severe external injuries, such as lacerations, exarticulations etc. may occur (PAUCOT 1901, CARVER and DINSLEY 1919, LOGAN 1939, ZUCKERMAN 1940, and others). Such injuries seem very rare, however. The lack of external changes in pure blast injuries has been pointed out in clinical cases by GAUDIN (1887), RAVAUT (1915), SENCERT (1915), LANGDON-DAVIES (1938), LOGAN (1939), MITCHINER and COWELL (1939), LOCKWOOD (1940), HADFIELD *et coll.* (1940), HADFIELD (1941), O'REILLY and GLOYNE (1941), WILLIAMS (1942), THEIS (1943), FEARNLEY (1945), and COHEN and BISKIND (1946), and in experimental investigations on animals by CARVER and DINSLEY (1919), MAIRET and DURANTE (1919), ZUCKERMAN (1940, 1941), and others. Burns may occur, usually of the flash-burn type (URIE, 1904, WILSON and TUNBRIDGE 1943, and others). When the blast is caused by bombs, grenades or other projectiles pure blast injuries would most likely be comparatively rare, (GANADO 1943). In such cases they are mostly combined with injuries caused by primary and secondary splinters and contusion injuries. This may bring forth symptoms which do not exist in uncomplicated blast injuries.

The blast experiments dealt with in this study have always been arranged as far as possible, so as to avoid secondary external injuries. In spite of this, external injuries have, nevertheless, occurred in some cases.

In 40 of the 199 rabbits exposed to detonations in the field, parts of the fur and the hairs of the nose on the side facing the charge, have been singed. This has occurred in animals so close to the charge that they have been within the glowing ball of explosion gases. In most of these cases the injuries have been extremely slight, and in no case have actual burns been seen.

Of other external injuries haemorrhages from smaller superficial wounds have been noted in 13 cases, and subcutaneous haemorrhages in 15 animals.

These injuries have been caused by the animal being hit by small stones or other objects thrown up from the ground by the blast wave.

The haemorrhages which are mostly localized to the side of the animal facing the charge consist, in most cases, merely of petechiae, but in some cases more massive haemorrhages are observed. They vary in size from a few mm to approximately 5 cm in diameter. The external injuries in all these cases have been so slight that they are without any particular significance to the actual blast injury.

None of the animals exposed in the detonation chamber has obtained external injuries.

### B. Injuries to the ear drums.

As the difference, from a mechanical point of view, between the blast wave and an ordinary sound wave mainly consists of the very much greater energy and amplitude of the former, it is quite natural that the ear drums, so sensitive to the energy of the sound wave, can be damaged by the detonation. This has also been known for quite a long time from clinical investigations. TRUETA states, that from 208 cases of ear drum injuries, seen in the Spanish Civil War, 175 had been caused solely by blast. Other authors, who have described injuries to the ear drums due to explosions or detonations, are COWEL (1885), BAHIER (1905), FRASER and FRASER (1917), MARRIAGE (1917), HOOKER (1924), PASSE (1940), ALEXANDER (1941, 1943), WILLIAMS (1942), TUNBRIDGE and WILSON (1943), BARROW and RHOADS (1944), and COHEN and BISKIND (1946), among others. All according to the magnitude of the force, the injuries can vary from a slight reddening of the ear drum to total destruction of the content of the middle ear.

While some authors maintain, that injuries to the ear drum only occur at high blast pressure, KROHN (1941) considers, that inspection of the ear drums is of value in all suspected cases of blast injury.

The present author has carried out routine examinations post-mortem on the ear drums of a great number of rabbits exposed to detonations in the field and in enclosed spaces.

This inspection has been carried out with an ordinary auriscope (according to WAPPLER) after the auricle has been cut away close to the cranium.

Injuries to the ear drum are common already at quite low pressures. In field blastings a reddening of the ear drums occurs even at pressures  $< 0.5 \text{ kgf/cm}^2$ , and ruptures may occur at pressures  $< 1 \text{ kgf/cm}^2$ . At these low pressures the ear drum nearest the charge is injured, while at higher pressures injury generally occurs also on the opposite side.

In detonations in enclosed spaces injuries to the ear drum occur even at noticeably low pressures probably due to the fact, that the damaging effect is greater because of reflexes and standing waves in the room (BERNAL 1941). The changes in the ear drums usually affect both sides here, and sometimes they may be more pronounced on that side which is turned away from the point in which the shock wave started.

### C. Retrobulbar haemorrhages.

Haemorrhages in the retrobulbar tissue have been noted in several of the severely injured animals.

### D. Internal injuries.

Every organ in the organism can be injured by a shock wave, if this has great intensity enough (cf. ABBOT, DUE and NOSIK 1943, and others). Usually however, the injuries are localized to organs containing air or gas, particularly then to the lungs and gastrointestinal canal. Why the gas content in particular should play a part in bringing about injury has been briefly touched upon on page 22 above.

Injuries to certain internal organs will be described below, namely to the central nervous system, to the lungs and other thoracic organs, and finally to the abdominal organs.

#### 1. Injury to the central nervous system.

As pointed out already on page 8 various changes in the central nervous system were previously considered an essential part of the syndrome of blast injury. In most cases it was possibly only a question of functional changes without macroscopic verifiable organic lesions. The possible existence of microscopic changes in the different parts of the brain and spinal marrow must not be entirely ignored.

however. In many cases it has been possible to find definite injuries, first and foremost haemorrhages, in different parts of the nervous system (see MOTT 1916, MAIRET and DURANTE 1919, STEWART, RUSSEL and CONE 1941 and ASCROFT 1943, among others).

It has not been the purpose here to make a minute study of the occurrence of injury to the central nervous system in blast. The brains from 71 of the rabbits, however, have been examined macroscopically. The spinal marrow has been examined in a few cases only.

In many cases, particularly in the less severely injured animals, no pathological changes have been noted. In cases of severe injury, on the other hand, a more or less pronounced congestion of the meninges is seen. In some cases great blood clots have been found in the cisterns. Macroscopically noticeable haemorrhages in the substance of the brain or spinal marrow have not been found in any one case.

In some of the severely injured animals the brain was swollen and oedematous with flattened gyri and filled up sulci. In one severely injured rabbit which had been allowed to live for 48 hours after the detonation, the brain was intensely pale and oedematous. The brain matter was noticeably soft and limp, especially within the left hemisphere.

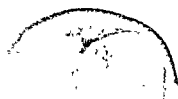
## 2. Injury to the lungs.

The predominance of the lung injuries in the syndrome of blast has already been pointed out.

It is not the author's intention here to give a full account of the lung blast injuries, as a number of extensive and first-rate surveys already exist on this subject, for instance by ZUCKERMAN (1940, 1941), KING and CURTIS (1943), TUNBRIDGE and WILSON (1943), WILSON and TUNBRIDGE (1943) and COHEN and BISKIND (1946). Only some complementary remarks may be given.

In the present material injuries to the lungs are, no doubt, the most common manifestation of blast injury. The relation between different pressure and impulse effects and the lung injuries, particularly the haemorrhages will be discussed more closely in a following chapter (chapter 11).

The general appearance and localization of the lung injuries agree,



on the whole, with those observed by other authors. This material confirms, in full, observations made by ZUCKERMAN and KROHN *et coll.* (1942), namely that haemorrhages in the lungs are mainly localized to the side turned towards the charge. This is seen clearly in animals which have been exposed to small charges at short distances. It seems, therefore, that this strongly supports ZUCKERMAN's opinion that the blast wave acts by direct impact on the body wall. For this speaks also the fact that the haemorrhages are mostly localized to those parts of the lungs which correspond to sinus phrenicocostalis (OSBORN 1940, ZUCKERMAN 1941) and costo-mediastinalis anterior (ZUCKERMAN 1941), consequently to those parts of the lung which become compressed and contused between the chest wall and the liver, on the one hand, and the chest wall and mediastinum, on the other. This localization occurs in the present material almost regularly in animals with moderate injuries, while it is seen less frequently in slightly injured and very severely injured animals. In the latter cases such arrangement of the haemorrhages is, of course, difficult to distinguish, as the whole lung is more or less hepatized. In these cases, however, a zone of darker, more massive haemorrhages is often seen along the lung margin.

According to ZUCKERMAN the haemorrhages are mainly localized to the central and lower parts of the lung, while the upper parts are more seldom affected. COHEN and BISKIND, on the other hand, could not find that any part of the lung was more often affected than the other.

In the present work localization of the haemorrhages to the upper parts of the lung is common even in cases with only slight or moderate injury. In a number of cases extensive haemorrhages existed throughout the upper lobes, while the rest of the lung was only slightly haemorrhagic. The haemorrhages often seem to follow the lines of the ribs. The existence of these "rib markings" appears to be of great interest from a pathogenic point of view, and will be discussed later (see chapter 10).

In cases of very severe lung lesion also bronchi and trachea may be filled with blood which gushes forth from the nose and mouth (PAUCOT, 1901, O'REILLY and GLOYNE, 1941, TUNBRIDGE and WILSON, 1943, WILSON and TUNBRIDGE, 1943, FEARNLEY, 1945, and others).

CARVER and DINSLEY (1919) often found bleeding from the nose

and mouth in rats and mice which had been placed so near the charge that they were within the area where the shock wave caused a crater ("zone of brisance"). In ZUCKERMAN's (1940, 1941) experiments such haemorrhages existed in all cases where the lung injury was severe enough to cause death, and also in a number of less severe cases.

As it seems evident from ZUCKERMAN's statement that bleeding from nose and mouth is a fairly constant sign of fatal lung injury, and, in consequence thereof, is an important prognostic sign, the existence of such haemorrhages have been studied in the present material.

The number of cases with haemorrhages from the nose are 38 out of 186 animals (21 per cent). The haemorrhages have mostly occurred in animals exposed to high pressure- and impulse values and in those which have obtained severe lung injuries. Only in one case (rabbit G 20) such damage has occurred in an animal which had been exposed to a maximum pressure  $< 5 \text{ kgf/cm}^2$ . The impulse in this case was great ( $1.34 \text{ gf} \cdot \text{sec/cm}^2$ ). In about half the number of cases the maximum pressure has been  $> 10 \text{ kgf/cm}^2$  and the impulse  $> 1 \text{ gf} \cdot \text{sec/cm}^2$ .

The risk of suffocation, owing to the respiratory passages being filled with blood, is of course great, and all animals with fairly extensive bleeding from the nose have died, most of them within 30 minutes after the detonation.

*Emphysema.* Traumatic emphysema of the lung in air blast injuries has been given only little notice in the previous literature. COHEN and BISKIND, however, mention, that an acute traumatic emphysema often exists, and that it is always of the vesicular type. It existed in their material both in parts with extensive but also with only slight haemorrhages. Interstitial emphysema was rare. In immersion blast injuries traumatic emphysema is common (see CAMERON *et coll.* 1942 and CLEMEDSON 1948) and nothing speaks in favour of emphysema of the lung being more common in immersion blast than in air blast. CAMERON *et coll.*, in serial sections of blasted lungs, have been able to show that haemorrhages and emphysema can exist separately and quite independently of each other. Both emphysematous and atelectatic areas can be seen in one and the same lung lobe. They are of opinion that the haemorrhages are the result of a less powerful force than the interstitial emphysema.

An acute traumatic emphysema and an acute distention of the lung are extremely common in the present material. Generally there is a diffuse enlargement of the lung, which in the most severe cases can be quite considerably enlarged. This lung is very much lighter in colour than normally. Often larger or smaller well demarcated emphysematous bullae are seen which contrast sharply against the surrounding lung tissue. Sometimes a marked bullous emphysema may coincide with a diffuse distention of the lung, but usually this is rare. In the less severe cases the emphysema appears mainly in the form of an increase of the slight marginal emphysema which is quite common even in normal rabbits.

*Pulmonary oedema.* COHEN and BISKIND (1946) found, that a pronounced pulmonary oedema was almost as common as haemorrhages and emphysema in air blast. The lung parenchyma surrounding the haemorrhages was diffusely light red and noticeably oedematous. Otherwise it was especially those parts of the lung that were free from haemorrhages, which were the formation sites of oedema. Also THEIS (1943) points out, that oedema of the lung may occur, and KROHN *et coll.* (1942) are of opinion that animals which die some time after the detonation, usually die from lung oedema.

In other respects, this question, so important from a theoretical, and to some extent even practical point of view, seems to have been given only little interest earlier. It is worth mentioning here, however, that OHLSSON (1947), who has studied the effect of oxygen treatment in blast injury, found a pronounced lung oedema after this treatment, while lung oedema hardly occurred in blasted animals not treated with oxygen.

The weight increase of a lung injured by blast, as already mentioned in chapter 8, can be considered to be due mainly to the haemorrhage. But it can, of course, also be due to the existence of a lung oedema, which may possibly occur as a direct effect of the blast trauma, or be a secondary reaction caused by the haemorrhages.

In order to get an idea whether lung oedema occurs to any larger extent, the amount of blood in the lung determined by weighing has been compared with the blood content calculated from determinations of the iron content of the lung according to the method described on page 39. By simultaneous determination of the iron content of the lung and of blood from vena cava a measure is obtained of the

quantity of blood, which corresponds to the amount of iron found in the lung. No importance has been attached to the content of tissue-bound iron in the lung. On the other hand, the values obtained from the determination of iron have, prior to the comparison, been reduced by the mean value of the normal blood content of the lung, obtained by determinations on a number of control animals.

Determinations of the blood iron in 15 normal rabbits gave a mean value of  $0.31 \pm 0.03$  mg/ml ( $\sigma = 0.11$ ). In the same animals the iron content in left lung was approximately  $0.26 \pm 0.04$  mg ( $\sigma = 0.14$ ) and in right lung  $0.32 \pm 0.03$  mg ( $\sigma = 0.11$ ), which corresponds to 0.8 and 1.1 ml of blood in left and right lung, respectively, or 0.2 and 0.3 ml of blood per g of lung tissue, respectively.

The blasted animals have been divided into two groups, one including animals which have died or been killed within 2 hours after the detonation, and the other animals which have lived for a longer period — up to 4 days.

The mean value of the blood content obtained by iron determination and by weighing is for the former group ( $< 2$  hours)  $1.27 \pm 0.33$  ml and  $1.93 \pm 0.50$  ml, respectively, for the left lung and  $5.33 \pm 1.0$  ml and  $7.81 \pm 1.38$  ml, respectively, for the right lung. In the group of animals which have lived more than two hours the values are  $1.22 \pm 0.42$  ml and  $1.60 \pm 0.27$  ml, respectively, for the left lung and  $3.15 \pm 0.52$  ml and  $5.63 \pm 0.97$  ml, respectively, for the right lung.

In almost all animals the weight increase of the lungs is greater than that which corresponds to the quantity of blood obtained by the determination of iron. This difference, which is much greater in the more severely injured lung, would, therefore, point to the existence of a lung oedema, more pronounced in the more severely injured lung which has been turned towards the charge.

In order to decide whether this difference is significative or perhaps only caused by chance, statistical treatment with *t*-analysis has been carried out. It is then found that no statistical difference exists as regards the left lungs neither in animals which have lived less than two hours ( $0.4 > P > 0.3$ ) nor in those which have been alive for a longer period ( $0.5 > P > 0.4$ ). As regards the right lungs the difference is probable ( $0.05 > P > 0.02$ ) in that group of animals which have lived more than 2 hours, while no statistical difference exists in animals which have died or been killed within that time ( $0.2 > P > 0.1$ ).



### 3. Injury to other thoracic organs.

Injury to the *heart* seems quite rare but has been described, nevertheless, by PAUCOT (1901), DELACROIX (1907), RUSCA (1915), THOMAS and JOHNSON (1915), STEWART, RUSSEL and CONE (1941) and COHEN and BISKIND (1946), and others. SHELLEY and HORWOTH (1946) observed air bubbles in the pericardium after the detonation.

EDELMAN, WHITEHORN, LEIN and HITCHCOCK (1946) mention that heart, lungs and brain are the most common localizations of haemorrhages caused by explosive decompression.

ZUCKERMAN (1941) observed haemopericardium in one animal. He states that a dilatation of the right ventricle was common in animals which died suddenly owing to blast. On the other hand, no microscopic changes existed in the myocardium or endocardium after blast.

A moderate dilatation of the right ventricle is fairly common in the present material, especially in the severely injured animals. In one case a violent aneurysmatic dilatation of the right ventricle was observed. On the other hand, macroscopic lesions have never been found in the heart. *Microscopic examination of the myocardium shows, however, that ruptures of the muscle fibres and small haemorrhages between these are common in animals suffering from severe blast injury.*

Haemorrhages in *thymus* and in *mediastinum* occur, but do not seem particularly common.

*If summarizing the results we find that pulmonary injuries including haemorrhages and traumatic emphysema are the most common changes in blast injury. Pulmonary oedema may occur but seems not to be a very important factor. Blood in the upper respiratory passages and bleeding from nose and mouth indicates a very severe, usually mortal lung injury.*

*Macroscopically detectable injuries to other thoracic organs may be seen but are not common. In severe blast injury, however, ruptures of muscle fibers and haemorrhages in the myocardium are common.*

### 4. Injury to abdominal organs.

According to the literature, damage to abdominal viscera seems proportionally rare in air blast. ZUCKERMAN (1941) and KROHN (1941), however, found in their experimental investigations that

lung- and abdominal blast injuries were combined in about 40 per cent of the animals. Two thirds of those animals, which had obtained abdominal injuries, had been killed by the detonation. Krohn mentions, however, that more serious abdominal injuries were rare.

As in immersion blast, the injuries are mainly localized to the gastro-intestinal canal, especially to those parts containing gas, where both haemorrhages and perforations may occur. Haemorrhages in solid organs would probably be rare, but have been described, for instance in the *liver* (RUSCA 1915, ZUCKERMAN 1941, LATNER 1942, TUNBRIDGE and WILSON 1943, SHELLEY and HORWATH 1946) *pancreas* (LATNER 1942) *spleen* (RUSCA 1915, ZUCKERMAN 1941, COHEN and BISKIND 1946) *kidneys* (RUSCA 1915, MAIRET and DURANTE 1917, 1919) *adrenals* (ZUCKERMAN, and COHEN and BISKIND) and *testes* (COHEN and BISKIND). *Retroperitoneal* haemorrhages have been described by WILLIAMS (1942), and LATNER (1941), in an experimental study of the effects caused by a sudden suction wave, found haemorrhages in the *fallopian tubes* in one case. Lesions in organs with liquid content are rare, but have, nevertheless, been described. PAUCOT (1901), thus, found in a man injured by an exploding dynamite blasting-cartridge a small rupture of the gall bladder. ZUCKERMAN (1941) describes a case of rupture of the bladder.

In the present material abdominal lesions are very common as compared with statements in the earlier literature. It may be pointed out already here, however, that the frequency of these lesions, first and foremost, of course, depends upon the intensity of the blast wave to which the animals have been exposed.

In 191 rabbits examined, abdominal lesions were found in 116 animals (61 per cent). Most common were haemorrhages in the gastro-intestinal canal, and perforation of the intestine existed only in 20 animals (= 17 per cent of the animals having abdominal lesions).

Table 4 below states the number of cases of haemorrhage and perforations within different parts of the gastro-intestinal canal and other abdominal organs.

The haemorrhages in the gastro-intestinal canal preponderate quite considerably over those in other abdominal organs. In respect to frequency the different parts of the gastro-intestinal canal are affected in the following order:

TABLE 4. Localization and frequency of abdominal lesions.

Localization	Number of cases	Per cent of all rabbits used	Per cent of rabbits with abdominal lesions
<i>1. Gastro-intestinal canal.</i>			
<i>Haemorrhage.</i>			
Stomach.....	2	1	1.7
Small intestine.....	29	15	25
Coecum .....	63	33	54
Appendix.....	9	4.7	7.8
Colon .....	65	34	56
<i>Perforations.</i>			
Stomach.....	0	0	0
Small intestine.....	5	2.6	4.3
Coecum .....	11	5.8	9.5
Appendix .....	0	0	0
Colon .....	7	3.7	6
<i>2. Other organs.</i>			
<i>Haemorrhage.</i>			
Liver .....	9	4.7	7.8
Pancreas .....	3	1.6	2.6
Spleen .....	1	0.5	1
Kidneys .....	10	5.2	8.6
Bladder .....	8	4.2	7
Retroperitoneal .....	13	6.8	11

Upper and lower part of colon,

Lower part of coecum,

Middle part of colon,

Middle and upper part of coecum,

Upper, middle and lower part of the small intestine,

Appendix vermiformis,

Stomach.

In an experimental study on immersion blast injuries in goats, CAMERON, SHORT and WAKELEY (1943) found that the various parts of the intestinal canal with regard to frequency were affected in the following order: middle and lower part of colon, upper part of colon, upper part of the small intestine, middle part of the small intestine, lower part of the small intestine. The lesions in colon preponderated

over all. The differences in frequency in their material and in this may be due to several different reasons. The species of animal, for example, may be of importance. The lesions also depend to a large extent on the condition of the gastro-intestinal canal at the moment of the detonation (whether it is filled with gas or solid content etc.). Possibly certain differences in a blast wave in air and in water may also play a part, though this seems more improbable. In more than half the number of cases with lesions in the gastro-intestinal canal this has shown an abnormal gas content. The lower part of colon has often been filled with gas. Within this area striated haemorrhages in the intestinal wall have often been observed.

*It may be concluded, then, that abdominal injuries are a common result of blast, as of all rabbits exposed, 61 per cent have obtained haemorrhages or perforations in different abdominal organs. Gas-containing viscera are the most vulnerable while solid parenchymatous and fluid-filled organs are more seldom affected. No pathological changes have been found in the adrenals.*

## CHAPTER 10.

### The Occurrence of "Rib Markings" in Lung Blast Injury.

It has been pointed out previously that the lung haemorrhages, except in slight cases, are mainly localized to the parts of the lungs, corresponding to sinus phrenico-costalis and costo-mediastinalis anterior. In many cases another arrangement of the haemorrhages is observed, which is specially interesting from a patho-physiological point of view, namely, that they seem to follow the projection of the ribs on the lung surface.

This arrangement of the superficial haemorrhages of the lung has been observed post mortem in persons injured by blast, by OSBORN (1941) and HADFIELD (1941). In 12 cases examined by WILSON and TESHNUPAR (1943) such "rib markings" were found, and these authors maintain, like Hadfield, that "rib markings" were most common in children.

Also in animal experiments "rib markings" have been found both in air blast and in immersion blast. ZUCKERMAN (1940, 1941) points

out that haemorrhages following "the lines of the ribs" were common in his experiments, even in cases where the haemorrhages affected almost the entire lung surface. In the most severe cases superficial lacerations of the lung tissue appeared along the ribs, which seemed to have been forced into the lung. Similar superficial ruptures were found by BARCROFT (1939) in goats. The ruptures were localized along the vertebrae and ribs reaching some 5 to 10 mm into the lung parenchyma. CAMERON, SHORT and WAKELEY (1942), who have studied the effects on goats of immersion blast, state that in animals at some distance from the charge and consequently with only moderate lung injuries, "rib markings" were seen in 50 per cent of the animals. In animals with severe lung lesion, they were not so common.

The "rib markings" are supposed to correspond to the ribs and should be due to the under-lying lung tissue being contused by the ribs. This could occur either by the ribs being forced into the lung by the shock wave (ZUCKERMAN), or by the shock wave passing through the yielding anterior abdominal wall thus forcing the diaphragm upwards, whereby the lung is forced upwards and outwards against the chest wall (WILLIAMS 1942). In this connection it should be pointed out, however, that "rib markings" can also be caused by rapid decompression without preceding high pressure (LATNER 1942).

The above explanation of the mechanism of the injury, however, is not generally accepted, and GREAVES *et coll.* (1943) are of opinion that the "rib markings" correspond to the interspaces rather than to the ribs. This they consider themselves having found in experiments on goats in which animal the ribs are comparatively narrow and the interspaces broad. COREY (1946) who, in his survey of blast injuries points out that "rib markings" are common particularly in immersion blast, emphasizes also that it is still not certain whether they are due to the ribs being forced inwards, or to a transmission of the shock wave through the interspaces.

A localization of the superficial haemorrhages producing the "rib markings," mentioned by earlier authors, is very common in the present material. "Rib markings" exist in all degrees of lung lesion from the most insignificant where small petechiae are arranged along the ribs (see fig. 19 a), to the very severe lesions where haemorrhages exist throughout the whole lung (see fig. 19 c).

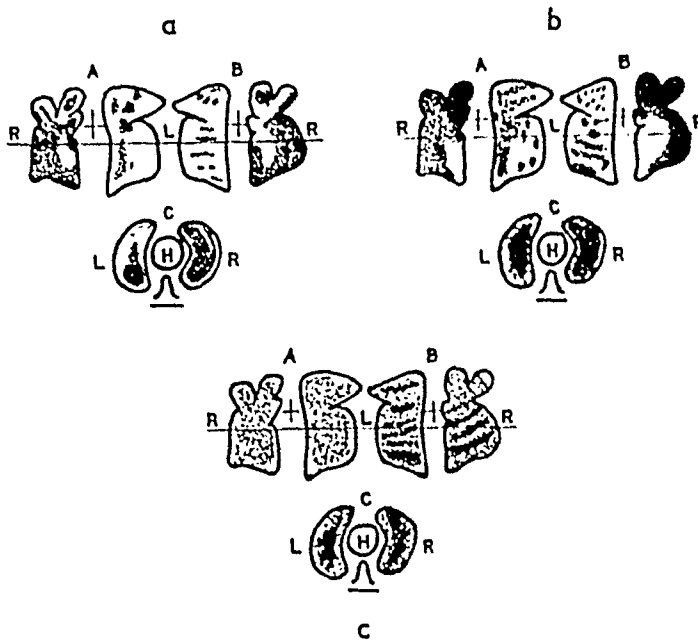


Fig. 19. "Rib markings." Schematic drawing of lungs with haemorrhages on the surfaces corresponding to the intercostal spaces. *a.* slight and *b.* moderate haemorrhages. *c.* "rib markings" in a lung with very severe haemorrhages. A = ventral and B = dorsal surface. C = cross-section. R = right and L = left lung. H = Heart.

The number of cases with "rib markings" and their frequency in the two lungs, are shown in table 5.

TABLE 5. Frequency of "rib markings" in the authors experiments.

Type of experiment	Number of animals	Number of cases with "rib markings"		
		Left lung	Right lung	Both lungs of the same animal
Experiments with small charges (weight < 2 kg).....	106	47 (44 %)	4 (4 %)	4 (4 %)
Experiments with big charges (> 2 kg).....	67	28 (42 %)	12 (18 %)	8 (12 %)
Experiments in detonation chamber .....	55	35 (64 %)	20 (53 %)	27 (50 %)

The table shows that "rib markings" in air blast mainly occur in that lung which has been turned away from the charge. This is clear specially in blastings with small charges (weight < 2 kg), but also in

blastings with greater weights of charge and at greater distances from the charge they occur in that lung in almost half the number of cases. Also in the experiments in the detonation chamber they have been proved more frequent in the lung which is turned away from the charge. Here the difference is only negligible, however, and in 50 per cent. of the cases "rib markings" occur in both lungs simultaneously.

Localization of the "rib markings" to that side which is turned away from the charge has not previously been described. As this seems important from a pathogenic point of view a closer study of the genesis of the "rib markings" has been made, and for this purpose it has first been examined whether they really correspond to the ribs or to the interspaces.

### **The genesis of the "rib markings".**

For the study of this problem the following method has been used.

The animals have been anaesthetized and killed in the usual manner after which the skin has been removed from the thoracic region. By means of a record syringe with a long, fine cannula a number of interspaces were punctured on either side, and a small amount of Indian ink was injected, so that a small black point on the lung marked the puncture. The lung was removed in the usual way, and the localization of the ink points in relation to the "rib markings" was determined. The amount of Indian ink injected has been so small that it has not noticeably increased the weight of the lung. Injection experiments have only been carried out in a number of animals where moderate and severe lesions could be expected. A pneumothorax which might interfere with the localization of the lung in relation to the interspaces, has not occurred in any case.

Experiments with Indian ink have been made on 34 animals exposed to blast in the field, and on 12 animals in experiments in the detonation chamber. Of the animals in the first group, 17 have had "rib markings" in the left lung and 10 in the right. In some cases "rib markings" have been missing or only faintly visible. These animals have been excluded. *In all cases except one (a left lung) the ink punctures have struck in the "rib markings" or, in some cases, in an extension line from these.* In the latter cases the "rib markings" have not been extended over the whole costal surface but only over

part of it why the ink points have come to be outside them. These cases have been omitted.

Of the animals in the second group, 8 have had distinct "rib markings" in the left lung, and 6 in the right. In all these cases the ink punctures have struck in the "rib marking" or in its extension.

*In 41 lungs in all with distinct "rib markings" and where ink experiments have been performed there was, thus, one case only where the ink points have struck between the "rib markings." It seems evident, therefore, that the supposition propounded by GREAVES et coll., namely, that they correspond to the interspaces and not to the ribs, must be correct.*

As regards the genesis of these "rib markings" it has generally been supposed that they were caused by a direct contusion effect when the ribs were pressed violently against the under-lying lung tissue (see ZUCKERMAN 1941). As the "rib markings" now correspond to the interspaces instead, and mainly occur on the side which is turned away from the detonation, this explanation would not prove correct. Neither can the opinion of GREAVES et coll., namely that the haemorrhages correspond to the interspaces as these offer less resistance than the ribs against the shock wave, explain satisfactorily why the "rib markings" occur on that lung in particular which is furthest away from the detonation centre. The contusion effect must be considered fairly insignificant on that side.

In regard to the above-mentioned the following explanation seems more plausible, in spite of the fact that even this has certain flaws. When the animal is struck by the shock wave this acts, according to ZUCKERMAN's theory, like an impact by a solid object. The lung turned towards the blast centre will then be exposed to direct contusion. If the animal is not quite fixed it will also be subject to an acceleration whose magnitude is dependent on the impulse, *inter alia*. Before the animal has been set in motion by the shock wave this, however, has already passed. This is clearly shown in the pictures in fig. 20, which were taken with a running speed of 5500 pictures per second, and show a rabbit exposed to the detonation of a charge of 1 kg of TNT. The charge is placed on the ground right beneath the animal which is lying on a mesh with its left side turned towards the charge. This side will be exposed to a violent compression, while the opposite side is still unaffected. *A powerful compression wave, which must be carefully distinguished from the shock wave transmitted through the organism, will propagate through the animal*



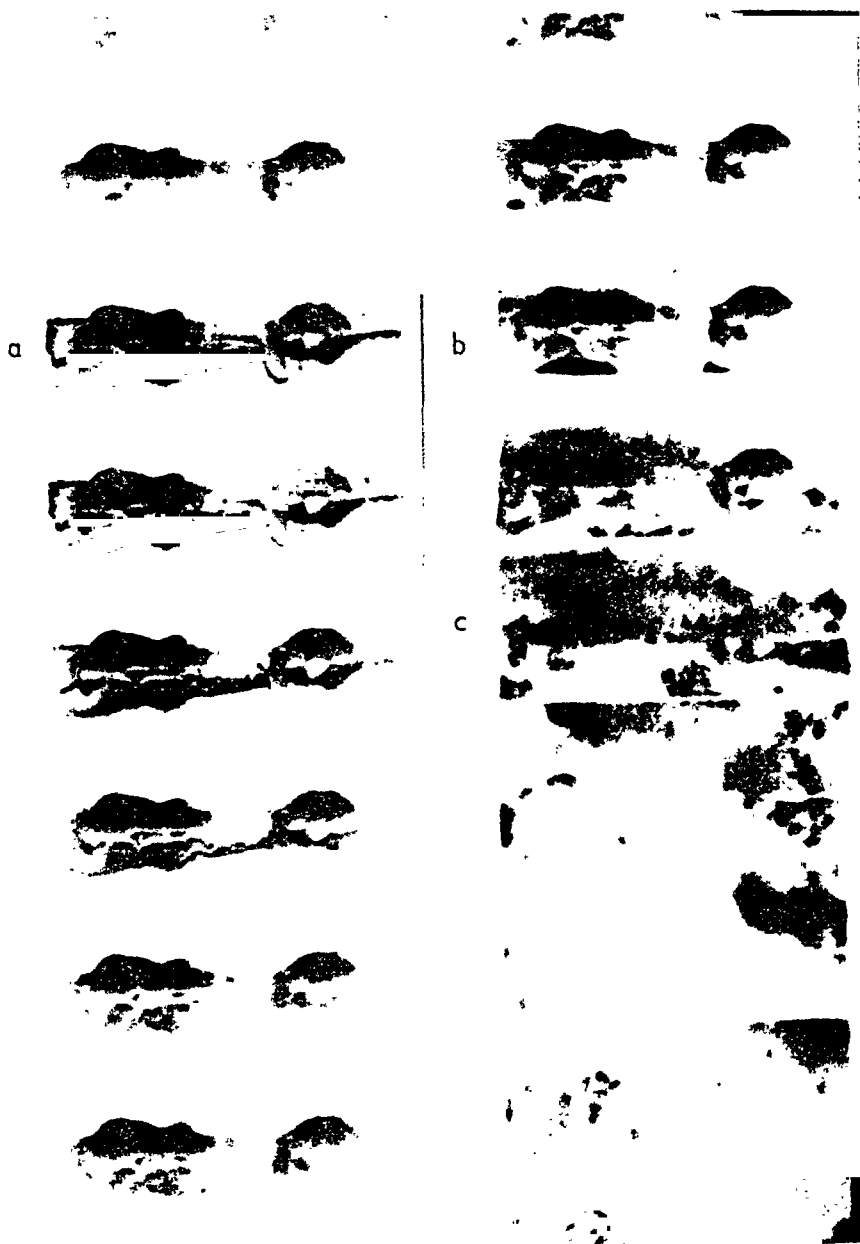


Fig. 20. High speed photograph of a rabbit exposed to a detonation of a charge of 1 kg of TNT. The rabbit is lying on a mesh with the charge 1.80 m below it. *a.* The animal is intensely illuminated when detonation starts. *b.* The shock wave has reached the animal. *c.* The animal is strongly compressed by the shock wave. Time of exposure: 50  $\mu$ s. Running speed: 5 500 pictures per sec.

*to its opposite side.* The compression is transmitted more rapidly through the ribs which consequently obtain greater speed than the soft tissues. This speed-difference, which will cause the intercostal

muscles and the lung tissue to be torn, will be greatest in the parts of the lung corresponding to interspaces. The haemorrhages in the intercostal muscles, sometimes seen in severe cases, may be the result of this tearing effect.

Experiments with impacts against the chests of anaesthetized animals have shown that lesions, exactly resembling lung blast, occur on the side which has been struck, and that haemorrhages may occur also in the lung on the opposite side.

*To summarize the results it may be pointed out, that an arrangement of the pulmonary haemorrhages forming the "rib markings" described by some authors, is very common. It has been shown by the present author, that the "rib markings" correspond to the costal interspaces and not, as generally believed, to the ribs. In experiments where the animals have been exposed side-on to the charge, the "rib markings" are most common in the lung on the opposite side, a fact which indicates that they cannot be the result of a direct impact on the chest wall. The present author is of opinion, that this special localization of the lung injuries is the result of extension strain forces caused by a sudden compression wave propagating through the animal.*

## CHAPTER 11.

### Relation between the Physical Qualities of the Blast Wave and the Extent of the Blast Injury.

Already quite early it was realized that the injuries to the organism caused by an explosion generally depended on the proximity of the injured to the explosion and on the force of the detonation.

In technical blasting experiments an attempt has been made to correlate the mechanical effect of the explosive with the magnitudes of the pressure and impulse of the blast wave. In experiments concerning blast injuries, on the other hand, such attempts do not seem to have been made, except in a few cases. The main reason for this may possibly be found in the fact that the principal part of the study of blast injuries has been carried out during the last world war when the object was to make clear, as soon as possible, the pathological peculiarities of these injuries, and first and foremost their

prophylaxis and therapy, while investigations of more theoretical interest had to take second place.

The possibility to obtain in clinical examinations of those injured by blast, reliable information as to the pressure and impulse that acted on the injured person at the moment of the detonation, must, naturally, be considered very slight. In this connection may be mentioned, however, the story related by MOTT (1916) of the aneroid barometer examined by the French engineer Arnoux and which was found in the pocket of a soldier injured by blast. The barometer is said to have shown that the man had been exposed to a pressure of  $> 10.000 \text{ kg/m}^2$ .

The fact that the human organism is remarkably resistant to violent pressure increase was often observed during the last war. Thus BERNAL (1941) points out in an excellent survey: "The Physics of Air Raids" that while a maximum pressure of  $0.07\text{--}0.7 \text{ kgf/cm}^2$  is sufficient to break most windows, a pressure rising to about  $7 \text{ kgf/cm}^2$  (i. e. an excess pressure of 6 atmospheres) is necessary for a human being to be endangered. This occurs only in the closest vicinity of the charge, and consequently, according to BERNAL, only very few people are injured directly by blast. WILLIAMS (1942) on the other hand maintains that the highest pressure a human being can endure without loss of efficiency is  $0.175 \text{ kgf/cm}^2$ . BORIE (1947), considers that this value lies at about  $0.35 \text{ kgf/cm}^2$ , while a static pressure of about  $6 \text{ kgf/cm}^2$  would be fatal. It can be mentioned, finally, that PHILIPS (1947) in his discussion on pneumonia after non-penetrating lung injury, states that a sudden blast pressure against thorax of more than  $0.175\text{--}0.525 \text{ kgf/cm}^2$  may cause injury to the lung.

COREY (1946) mentions that in a disaster with exploding mines in U. S. A. where several men had been so close that they obtained third degree burns, there were no blast injuries, and that investigations into air blast injuries had been abandoned in America, owing to their supposed lack of importance.

In earlier animal experiments a more exact measuring of the pressure seems to have been carried out only on a very small scale. Generally only the distance from the charge at which the animals had remained uninjured or had obtained certain injuries (for instance slight, severe and fatal injuries), has been mentioned. Even these statements are quite valuable, however, especially in such cases where also the kind and weight of the explosive have been stated. With the help of this information it is possible to get a certain idea as to the magnitude of the maximum pressure.

As far as can be judged from the literature the impulse of the blast wave has not been measured earlier in animal experiments in air blast, and in immersion blast experiments only on a limited scale (WAKELEY 1945).

One of the first to study the physiological effects of rapid changes

of pressure, though not in connection with blast experiments, however, was PAUL BERT (1878). He states that an excess pressure of 6 atmospheres followed by a rapid decompression caused nervous symptoms of different kinds. At an excess pressure of 8 atmospheres or more the experimental animals (cats and dogs) obtained injuries which rapidly led to death. Cats exposed to an excess pressure of 10 atmospheres followed by a rapid decompression, showed nervous symptoms (paraplegiae and paralysis) but, on the other hand, no haemorrhages in lungs or spinal marrow.

HOOKE (1919, 1924) in experiments where anaesthetized dogs were exposed to the pressure field around the muzzles of guns and howitzers, found that an excess pressure of about 20 atmospheres caused haemorrhages in the lungs. He could state, further, that while an excess pressure of about 20 atmospheres gave rise to shock in several of the dogs placed in front of a 10 inch gun, 27 atmospheres were not sufficient to cause shock in any animal exposed in front of a 12 inch howitzer. HOOKE explains this extraordinary result by stating that maximum pressure alone does not give rise to shock. The duration of the pressure rise is of great importance, he says, and in cases without shock the pressure has been of too short duration to overcome the physical resistance of the tissues. In all these cases there were haemorrhages in the lungs, but no other injuries.

The most satisfactory and exhaustive data concerning the effects of the blast wave on different experimental animals (mice, rats, guinea pigs, rabbits, cats, monkeys and pigeons) have been supplied by ZUCKERMAN (1940, 1941). In experiments with charges of about 32 kg H. E. none of the animals, placed more than 5.5 m from the charge, was killed, and no injuries were sustained at a distance of more than about 15 m. At distances of between 4 and 5.5 m almost all animals were killed. Piezo-electric determination of pressure gave the following values in the different distances:

Distance:	4	5.5	15 m.
Pressure:	8.8	4.4	0.44 kgf/cm <sup>2</sup> .

At the distance of 15 m the negative pressure in the suction wave was 0.09 kgf/cm<sup>2</sup>.

ZUCKERMAN further points out as regards rabbits, that no injuries resulted from a pressure of 0.35 kgf/cm<sup>2</sup>, but that the animals

could be killed by a pressure of  $3.5 \text{ kgf/cm}^2$ , and that  $7 \text{ kgf/cm}^2$  was sure to cause fatal injuries.

These statements are somewhat in contrast to a number of more recent investigations made by American scientists. Thus HORWATH and SHELLEY (1946) mention that a goat placed at a distance of about 4 m from a 100 m long charge of TNT weighing more than 2 000 kg and lying on the ground, probably had obtained no injuries of any importance. No data as to the maximum pressure are available in this case, but an estimate shows that it must have exceeded  $20 \text{ kgf/cm}^2$ .

COREY (1946) states that rabbits and guinea pigs at a distance of 30 m from a detonating charge of almost 800 kg of TNT were quite uninjured, and he found in blast experiments with small charges (0.23—9 kg of TNT) that animals immediately outside the flash area were unhurt, while those which were within this zone and had obtained burns, always had blast injuries.

GREAVES *et coll.* (1943) point out that if the organism is struck by a shock wave with a pressure of over  $35 \text{ kgf/cm}^2$  this is transmitted through the tissues to the lungs and gastro-intestinal canal, and DRAEGER, Barr and SAGER (1946) consider a pressure of this magnitude great enough to cause fatal ruptures of the pulmonary vessels or perforation of the intestinal canal. According to COREY (1945) a pressure of  $17.5 \text{ kgf/cm}^2$  should be sufficient to cause rupture of the intestines.

As it seems to be of great interest to study closer the correlation between maximum pressure, impulse and blast injury, and because the earlier statements on this problem are rare and incomplete, some attention will be given to it here.

The material consists of 176 rabbits *exposed to detonations in the field* and which have died or been killed within 48 hours after the detonation. Animals which have lived for a longer time than 48 hours have not been included, as it is difficult to estimate in these the extent of the original injury.

The study of the correlation between the physiological effect and the pressure and impulse of the shock wave has been carried out in respect to lung injuries, especially the haemorrhages, and to abdominal injuries. In the discussion below an account will be given of lesions caused by different pressure- and impulse values, after

which follows an analysis of the part played by the different physical components in causing these injuries.

## 1. Lung injury.

As already mentioned previously the study of blast injuries during the last war has clearly shown that the earlier opinion of blast injuries being mainly a neurologic problem, is wrong, and it has been made clear that the lung lesions are the predominating factor in the blast syndrome.

The present author has found in a great number of blast experiments carried out earlier both in the field and in confined spaces and under the most varying experimental conditions and with a material consisting of more than 600 rabbits, that the lung lesions, particularly the haemorrhages, are quite closely related to the maximum pressure of the blast wave.

### a. The significance of the maximum pressure.

Already at quite low maximum pressures small punctiform haemorrhages appear in the lungs. These occur particularly in detonations in confined spaces, but even in field blastings remarkably low pressures are often enough for such changes to take place. In a number of rabbits they have occurred already at a max. pressure of about  $0.5 \text{ kgf/cm}^2$  in the field, and at one tenth of this pressure in confined spaces.

These changes, which are capillary haemorrhages within regions of the lung, which, for some reason or other, are less resistant, are generally localized immediately below the pleura. Haemorrhages in the deeper parts of the lungs are uncommon at these low pressures. Petechiae may be found anywhere on the surface of the lung, without predilection for any particular region. The interlobar surfaces, however, are generally free from petechiae.

*At maximum pressures  $< 3 \text{ kgf/cm}^2$  lung haemorrhages do not appear consistently, and if they occur no relation seems to exist between the maximum pressure and the extensiveness of the haemorrhages. Nor is there any significant difference between the extent of the haemorrhages in the two lungs. They might even be more dominant on the side which is turned away from the charge. Thus, rabbit S 8,*

for instance, which was exposed to a pressure of 2.68 kgf/cm<sup>2</sup>, had some small haemorrhages in the left lung, while the right lung was quite uninjured.

*At maximum pressures > 3 kgf/cm<sup>2</sup> pulmonary haemorrhages occur regularly on the side turned towards the charge, and it is usually a rule here that the higher the pressure the more severe are the lung injuries.* It should perhaps be pointed out, however, that no strictly linear relation is obtained, as, of course, quite a number of other factors may be of importance, for instance, the general condition of the animal.

At very high pressures (> 10 kgf/cm<sup>2</sup>), which, often cause injuries rapidly leading to death, the quotients of weight increase for the lungs are often dependent on whether the animal suffered an immediate death or whether it has lived for a longer period. If it has not died immediately the quotient is usually greater as the bleeding continues for some time after the detonation.

The correlation between the maximum pressure of the shock wave and the lung lesions is shown in tables 6 and 7. Only the haemorrhages have been used as an index of the extent of the blast injury, since the volume increase of the lung caused by the detonation does not appear to have any significant relation to the force of the blast wave. The lung haemorrhages have been classified in the following way:

*Very slight injury:* only single petechiae.

*Slight injury:* great numbers of petechiae or a few somewhat larger haemorrhages mainly on the surface. Quotients of haemorrhage < 1.20.

*Moderate injury:* more widely spread, though not massive haemorrhages. Quotients = 1.20—1.50.

*Severe injury:* widely spread, massive haemorrhages. Quotients > 1.50.

The limits between the groups of pressure values have been chosen arbitrarily.

Table 6 shows the correlation between maximum pressure and haemorrhages in the lung nearest the charge. At maximum pressures < 3 kgf/cm<sup>2</sup> the haemorrhages are, for the most part, very slight. Severe lesions do not exist in this group and none of the 24 animals have been killed by the detonation. Even the 23 animals exposed

TABLE 6. Extent of pulmonary injuries (haemorrhages) as a function of the max. pressure of the shock wave.

*Right lung.*

Max. pressure kgf/cm <sup>2</sup>	Num- ber of ani- mals	No injury	Very slight injury	Slight injury (quot. <1.20)	Moderate injury (quot. 1.20— 1.50)	Severe injury (quot. >1.50)	Animals killed by detonation. ( )=died more than one hour after the detonation
0— 2.99	36	8	19	5	4		
3— 4.99	28		13	7	4	4	1+(1)
5— 7.99	25			10	5	10	3+(1)
8— 9.99	20			2	5	13	3
10—14.99	45			5	7	33	10+(3)
15—20	12					12	5+(1)
> 20	8					8	5+(1)

to maximum pressures of between 3 and 5 kgf/cm<sup>2</sup> have mainly very slight or slight injuries but also a number of cases with moderate and severe lesions occur in this group. Two of the animals belonging to this group died from the injuries, one immediately after the detonation, the other after a lapse of about 5 hours. The pressure values for these two animals were 4.5 and 4.9 kgf/cm<sup>2</sup>, respectively.

At maximum pressures > 8 kgf/cm<sup>2</sup> the great majority of the animals are severely injured, and at pressures > 15 kgf/cm<sup>2</sup> all animals are severely injured.

The number of animals dying as a result of the blast injuries increases rapidly with increasing pressure. Three animals out of 15 (= 20 per cent), exposed to maximum pressures of 8—10 kgf/cm<sup>2</sup>, died. In the group of 10—15 kgf/cm<sup>2</sup> approximately one third died (12 out of 39 = 31 per cent) and in the group of 15—20 kgf/cm<sup>2</sup> approximately half the number (6 out of 14 = 43 per cent). Finally, all animals exposed to a maximum pressure > 20 kgf/cm<sup>2</sup>, died.

Table 7 contains a similar survey of the pulmonary haemorrhages on the side turned away from the charge. Also here a relation between the maximum pressure and the extent of the injury exists. The haemorrhages, however, are generally less extensive on this side than on the side facing the charge, a fact which is shown even more clearly in table 15 below (page 90).



TABLE 7. Extent of pulmonary injuries (haemorrhages) as a function of the max. pressure of the shock wave.

*Left lung.*

Max. pressure kgf/cm <sup>2</sup>	Number of animals	No injury	Very slight injury	Slight injury (quot. <1.20)	Moderate injury (quot. 1.20— 1.50)	Severe injury (quot. >1.50)	Animals killed by detonation. ( )=died more than one hour after the detonation
0— 2.99	36	13	17	4	2		
3— 4.99	28	1	15	5	5	2	1+(1)
5— 7.99	25	2	7	7	3	6	3+(1)
8— 9.99	20		1	9	6	4	3
10—14.99	45		2	12	8	23	10+(3)
15—20	14			7		7	5+(1)
>20	8			1		7	5+(1)

## b. The significance of the impulse.

The technical study of blast has proved that the maximum pressure alone is not a sufficient measure of the damaging effect of the blast wave. Also the duration of the pressure and the impulse would be of great importance. This should, of course, also apply to the physiological effects, but no analysis as to this seems to have been made earlier.

Tables 8 and 9 show the correlation between the impulse of the

TABLE 8. Extent of pulmonary injuries (haemorrhages) as a function of the impulse of the shock wave.

*Right lung.*

Impulse gf · sec/cm <sup>2</sup>	Number of animals	No injury	Very slight injury	Slight injury (quot. <1.20)	Moderate injury (quot. 1.20— 1.50)	Severe injury (quot. >1.50)	Animals killed by detonation. ( )=died more than one hour after the detonation
0—0.49	44	6	19	12	6	1	
0.5—0.99	45	1	2	12	11	19	4
1—1.49	51	1		11	7	32	11+(6)
1.5—1.99	12			2		10	1
2—3	17				3	14	9
>3	7				1	6	2+(1)

TABLE 9. Extent of pulmonary injuries (haemorrhages) as a function of the impulse of the shock wave.

*Left lung.*

Impulse gf · sec/cm <sup>2</sup>	Num- ber of ani- mals	No injury	Very slight injury	Slight injury (quot. <1.20)	Moderate injury (quot. 1.20— 1.50)	Severe injury (quot. >1.50)	Animals killed by detonation. ( )=died more than one hour after the detonation
0—0.49	44	13	21	8	1	1	
0.5—0.99	45	2	13	18	6	6	4
1.0—1.49	51	1	7	15	9	19	11+(6)
1.5—1.99	12		1	4	2	5	1
2—3	17			2	2	13	9
>3	7			1		6	2+(1)

blast wave and pulmonary haemorrhages caused. The classification of the lesions has been made in the same way as mentioned above.

At small impulse values ( $< 0.5$  gf · sec/cm<sup>2</sup>) the lesions are for the most part only very slight or slight. None of the 29 animals exposed to impulse values  $< 0.5$  gf · sec/cm<sup>2</sup> have obtained severe lesions or died as a result of the detonation. Already at impulses of between 0.5 and 1 gf · sec/cm<sup>2</sup>, however, almost half the number of animals (43 per cent) have been severely injured, and four have been killed by the detonation. Animals exposed to blast waves with an impulse  $> 1.5$  gf · sec/cm<sup>2</sup> have, in the majority of cases, obtained severe, often mortal lung lesions.

Consequently a certain correlation exists between the maximum pressure and the impulse of the blast wave, on the one hand, and the lung lesion caused, on the other. The strength of this correlation has been determined mathematically for the lung facing the charge in 83 rabbits exposed to blast in the field. *For the correlation between maximum pressure and lung injury the correlation coefficient  $r = 0.64 \pm 0.06$ , is obtained. For the correlation between impulse and lung injury  $r = 0.53 \pm 0.08$ , is obtained. The correlation, therefore, is good in either case, but is somewhat stronger for the correlation between maximum pressure and lung lesion.*

## 2. Abdominal lesions.

Hardly any equivalence to the close relation existing between the maximum pressure and impulse values and the extent of lung lesions

TABLE 10. This table shows the lowest max. pressure and impulse values at which injuries in different abdominal organs occurred.

Injury	Maximum pressure kgf/cm <sup>2</sup>	Impulse gf · sec/cm <sup>2</sup>
<i>Haemorrhage:</i>		
Stomach .....	6.3	1.76 *
Small intestine .....	1.8	0.6 *
Caecum .....	0.6	0.5 *
Appendix .....	1.24	0.24 *
Colon .....	1.7	0.38
Liver .....	1.0	0.95 *
Pancreas .....	2.68	0.26 *
Spleen .....	6.3	1.76 *
Kidneys .....	1.35	0.43
Bladder .....	0.59	1.56
Retroperitoneal .....	2.5	0.68 *
<i>Perforations:</i>		
Small intestine .....	8.84	0.43 *
Caecum .....	2.5	0.62
Colon .....	8.9	0.51

\* Impulse values marked with \* have been obtained at the same blast as the max. pressure given in the corresponding place in the max. pressure column.

is seen, as far as the abdominal lesions are concerned. *Haemorrhages*, especially in the intestine, may occur relatively often at remarkably low pressure- and impulse values, while, on the other hand, many animals exposed to very strong blast effects and which have severe lung injuries, show none or only slight lesions in the abdomen, besides a more or less pronounced hyperaemia. A typical example of this is one animal, which, in spite of having been exposed to a shock wave with a maximum pressure of 20.9 kgf/cm<sup>2</sup> and an impulse of 1.36 gf · sec/cm<sup>2</sup>, had only a few small ruptures on the upper side of the liver. The reason for this is hard to explain, but one thing is certain, namely that the condition of the abdominal organs, i. e. in the extent to which they are filled with gas or solid content etc., is of very great importance.

Table 10 gives the lowest maximum pressures and the smallest impulse values at which lesions (haemorrhages, ruptures) have occurred in various abdominal organs.

TABLE 11. Frequency of intestinal haemorrhages at different max. pressure and impulse values.

Max. pressure kgf/cm <sup>2</sup>	Num- ber of ani- mals	Per cent of all rabbits examined	Per cent of rabbits with intest. haemor- rhages	Impulse gf · sec/cm <sup>2</sup>	Num- ber of ani- mals	Per cent of all rabbits exam- ined	Per cent of rabbits with intest. haemor- rhages
< 5	15	7.9	15	< 0.5	3	1.6	3
< 10	40	21	40	< 1	29	15.2	29
< 15	80	42	80	< 2	80	42	80
< 20	90	47	90	< 3	94	49	94
> 20	10			> 3	6		

A survey of the frequency of intestinal haemorrhages at different pressure- and impulse values is given in table 11.

Abdominal lesions in air blast have earlier been considered by several authors to be rare. Therefore it is surprising to find that they may occur even at relatively low maximum pressures. Thus 25 out of 116 animals (i. e. 21.5 per cent) with abdominal injuries have been exposed to a blast wave with a maximum pressure < 5 kgf/cm<sup>2</sup>. Of these 25 animals 15 had haemorrhages in the intestinal canal, as proved by table 11.

*The most vulnerable abdominal organ is coecum*, probably because of its considerable size in the rabbit and its proximity to the anterior abdominal wall as well as of its thin wall. In addition to this it is relatively often to a considerable extent filled with gas. Changes in the more thick-walled appendix are not so common. In one animal (G 59) a number of small haemorrhages were seen in the appendix which, because of a faecal stone, was totally shut off from the rest of the intestine. The maximum pressure and impulse in this case was only 1.24 kgf/cm<sup>2</sup> and 0.24 gf · sec/cm<sup>2</sup>, respectively.

*Haemorrhages in the stomach* existed in two animals. These had been exposed to a maximum pressure of 6.3 kgf/cm<sup>2</sup> and 15.5 kgf/cm<sup>2</sup>, respectively. In the first animal the lesion was only slight. The stomach of the second animal, on the other hand, showed an extensive haemorrhagic infiltration.

*Ruptures of viscera* are, with some single exception, seen only in animals exposed to high maximum pressure and relatively great impulse. This is clear from table 12, in which the means of the pres-

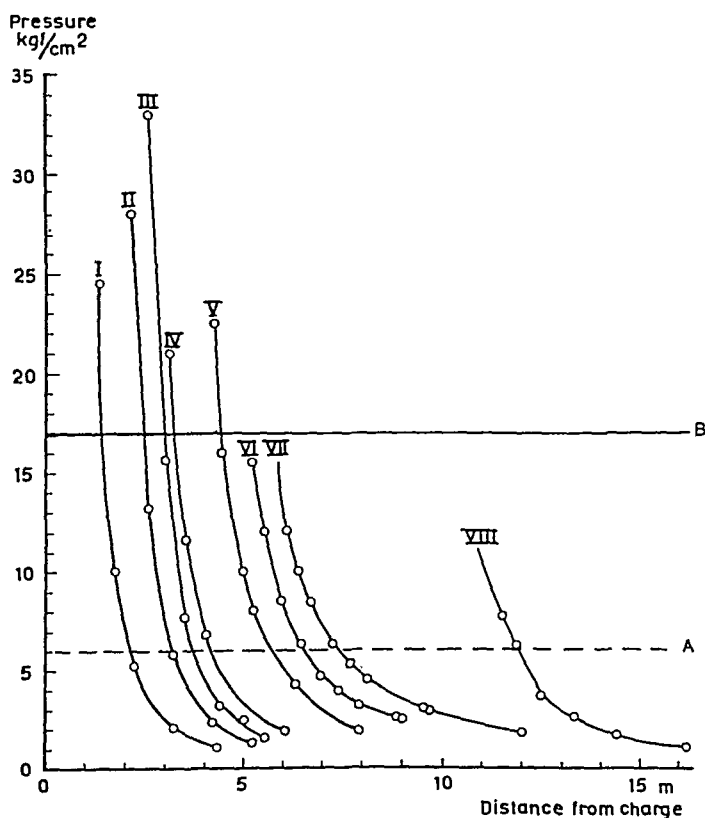


Fig. 21. Pressure-distance diagram. Lines A and B indicate the pressure values at which 50 per cent of the rabbits obtained severe lung injuries and mortal injuries respectively. Weights of charge: I: 1.25; II: 2.5; III: 3.75; IV: 5.0; V: 14.5; VI: 21.75; VII: 29.25; VIII: 100 kg.

sure- and impulse values sustained by animals with intestinal ruptures, have been classed together.

TABLE 12. Mean value of the max. pressure and impulse values obtained by rabbits which have received intestinal perforations.

Localization of injury	n	Max. pressure kgf/cm <sup>2</sup> $M \pm \varepsilon(M)$	Impulse gf · sec/cm <sup>2</sup> $M \pm \varepsilon(M)$
Small intestine .....	5	$15.20 \pm 4.27$	$1.37 \pm 0.40$
Coecum .....	11	$15.55 \pm 2.82$	$1.73 \pm 0.35$
Colon .....	7	$13.06 \pm 1.74$	$1.80 \pm 0.35$

**Injurious effect as a function of the distance from charge.**

The distance from a detonating charge within which severe injuries are obtained, is fairly small owing to the fact that the maxi-

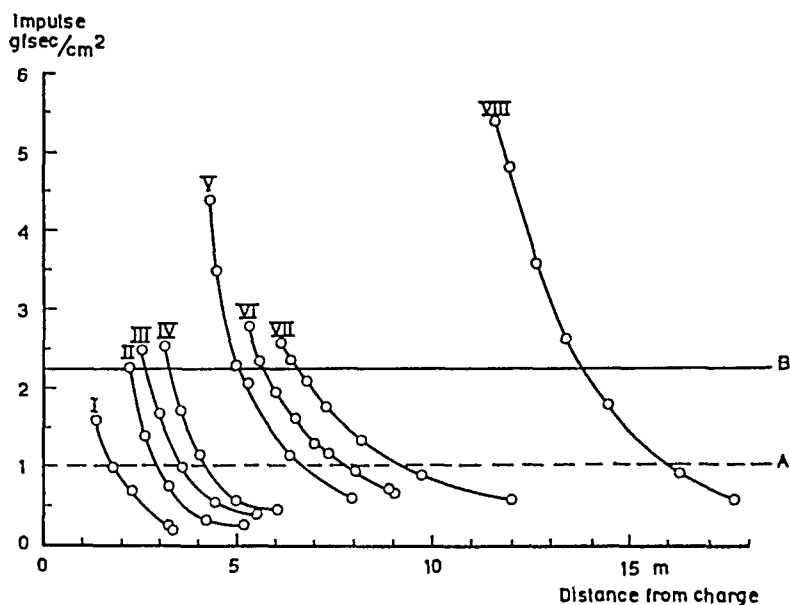


Fig. 22. Impulse-distance diagram. Lines A and B indicate the impulse values at which 50 per cent of the rabbits obtained severe lung injuries and mortal injuries respectively. Weights of charge as in fig. 21.

imum pressure and impulse decrease rapidly with increasing distance from the charge. This is shown in figs 21 and 22, which give pressure-distance and impulse-distance diagrams for some of the weights of charge used. Only when the pressure and impulse have decreased to quite low values at relatively great distance from the centre of detonation, the equalization of pressure occurs more slowly.

The lines A and B drawn in the diagrams run, in each of the pressure-distance and impulse-distance diagrams, through the pressure- and impulse values at which 50 per cent of the animals have obtained severe and fatal lung lesions, respectively.

As to the maximum pressure these values are 6 kgf/cm<sup>2</sup> and 17 kgf/cm<sup>2</sup>, and as to the impulse 1 gf·sec/cm<sup>2</sup> and 2.25 gf·sec/cm<sup>2</sup>, respectively. These values have been obtained from diagrams where all animals have been grouped in classes with regard to pressure- and impulse effect and with the class limits of 1 kgf/cm<sup>2</sup> and 0.25 gf sec/cm<sup>2</sup>, respectively. The distribution of the percental number of animals within each class has then been smoothed by introducing successive means (see for instance DAHLBERG, 1940). The values obtained are, of course, only approximate.

TABLE 13. Quotients of lung injury of rabbits exposed to high max. pressure and small impulse (Max. pressure  $>4$  kgf/cm<sup>2</sup>. Impulse  $<0.5$  gf · sec/cm<sup>2</sup>.)

Rabbit	Max. pressure kgf/cm <sup>2</sup>	Impulse gf · sec/cm <sup>2</sup>	Quotients of lung injury			
			Haemorrhage (+ oedema)		Increase of lung volume (emphysema)	
			Left lung	Right lung	Left lung	Right lung
S 3	$4.05 \pm 0.5$	0.43	0.73	0.58	0.72	0.64
S 4	$4.05 \pm 0.5$	0.43	0.75	0.74	0.79	0.77
S 11	$5.25 \pm 0.7$	0.38	0.95	1.34	1.16	1.37
S 12	$5.25 \pm 0.7$	0.38	0.80	0.84	0.76	0.85
S 31	$4.73 \pm 0.5$	0.35	0.76	0.81	0.88	0.94
S 32	$4.73 \pm 0.8$	0.35	0.74	0.79	0.94	1.16
S 33	$4.08 \pm 0.4$	0.31	0.83	0.80	0.95	0.94
S 34	$4.08 \pm 0.6$	0.31	0.71	0.70	0.82	0.80
S 37	$4.59 \pm 0.7$	0.38	0.88	0.90	0.80	0.88
S 38	$4.59 \pm 0.5$	0.38	1.25	1.25	1.02	1.03
S 39	$5.98 \pm 0.7$	0.46	1.06	1.29	1.25	1.44
S 40	$5.98 \pm 1$	0.46	1.33	1.17	1.03	0.99
S 45	$6.18 \pm 1.1$	0.49	0.68	0.69	0.71	0.66
S 46	$6.18 \pm 0.75$	0.49	0.96	1.18	0.99	1.20
S 114	$5.36 \pm 0.9$	0.34	1.05	1.0	0.98	0.95
S 115	$5.33 \pm 0.9$	0.37	0.91	0.95	1.0	0.95
S 116	$5.19 \pm 0.8$	0.42	1.20	1.32	1.01	1.31
S 172	$8.84 \pm 0.9$	0.43	1.02	1.23	0.98	0.95

In the discussion above of the correlation between pressure, impulse and degree of lesion no consideration has been given to the fact that a mutual relation exists, to some extent, between the maximum pressure and impulse. Thus it is generally the rule, that a high maximum pressure is combined with a relatively great impulse. This relation, however, is not stable but varies with the size of the charge. With the small charges (weight of charge  $< 1$  kg) used, the relation between maximum pressure and impulse is approximately like 10:1. With the greater weights of charge the impulse will be comparatively greater at a given distance, and in certain cases it can be as great, or even greater than, the maximum pressure.

The fact that the impulse effect is also important in causing various degrees of lesion, has hardly been given any attention earlier, as

TABLE 14. Quotients of lung injury of rabbits exposed to low max. pressures and great impulses (Max. pressure  $< 4$  kgf/cm<sup>2</sup>. Impulse  $> 0.5$  gf·sec/cm<sup>2</sup>).

Rabbit	Max. pressure kgf/cm <sup>2</sup>	Impulse gf·sec/cm <sup>2</sup>	Quotients of lung injury			
			Haemorrhage (+ oedema)		Increase of lung volume (emphysema)	
			Left lung	Right lung	Left lung	Right lung
G 25	1.75	1.78	1.03	1.18	1.05	0.92
G 26	2.5	2.65	1.19	1.26	1.21	1.14
G 27	3.7	3.6	2.12	1.37	1.52	1.47
G 43	2.9	1.26	0.91	1.02	1.0	1.07
G 44	1.26	1.39	0.94	0.99	0.88	0.91
G 45	0.82	1.47	0.85	0.89	0.76	0.89
G 46	1.25	1.4	0.71	0.78	0.84	0.83
G 48	1.7	1.35	1.09	1.24	0.97	0.97
G 49	0.59	1.56	0.97	1.00	0.96	0.99
G 50	2.2	1.30	0.91	0.99	1.01	1.01

already mentioned. HOOKER (1924), however, has pointed out the importance of *the duration of the pressure*, and WAKELEY (1945) states, concerning lesions due to immersion blast, that neither the maximum pressure nor the impulse alone can determine the degree of damage. That this holds good also in air blast is clear from tables 13 and 14. Table 13 gives a survey of the lung lesions (quotients of haemorrhage and volume increases) in 18 animals exposed to blast waves with relatively high pressure ( $> 4$  kgf/cm<sup>2</sup>) but small impulse ( $< 0.5$  gf·sec/cm<sup>2</sup>). None of these animals have obtained lung lesions of any significance. On the other hand (see table 14), severe lung lesions have been found only in one animal (G 27) out of 10 exposed to relatively low maximum pressure ( $< 4$  kgf/cm<sup>2</sup>) but great impulse ( $> 0.5$  gf·sec/cm<sup>2</sup>). A high maximum pressure combined with a great impulse, on the other hand, will cause severe lesions, as shown in table 15.

A further analysis of the part played by the maximum pressure and impulse in causing total injury, is quite difficult for several reasons, one being the mutual relation of the maximum pressure and impulse, another the relatively great errors of the pressure- and impulse measurements.



TABLE 15. Severe lung injuries (great quotients) of rabbits exposed to high max. pressures and great impulses.

(Max. pressure  $>4$  kgf/cm<sup>2</sup>. Impulse  $>0.5$  gf·sec/cm<sup>2</sup>.)

Rabbit	Max. pressure kgf/cm <sup>2</sup>	Impulse gf · sec/cm <sup>2</sup>	Quotients of lung injury				Comments
			Haemorrhage (+ oedema)		Increase of lung volume (emphysema)		
			Left lung	Right lung	Left lung	Right lung	
S 42	13.2±2.4	0.79	1.81	3.63	1.88	2.39	Died after 8 min
S 110	11.5±1.3	1.0	2.22	3.40	1.37	1.68	
S 113	12.3±2	0.62	1.53	3.05	1.03	0.90	
S 124	20.6±3.3	1.1	1.91	3.82	1.43	0.89	
S 126N	15.8±2.5	0.95	1.15	3.22	1.93	2.04	
S 134	18.6±2.8	1.4	3.87	4.77	1.59	1.63	Died immediately
S 135	16.5±2.5	1.1	1.86	3.42	1.39	1.09	Died after about 12 ho
S 137	13.3±1.9	1.13	2.14	3.75	1.66	1.97	Died after 10 min
S 140	12.5±1.8	1.15	2.16	3.09	2.00	2.00	Died immediately
S 149	13.3±2	1.4	2.10	3.67	1.47	1.30	Died after about 5 hours
S 152	20.2±2.9	1.25	2.00	3.54	1.38	1.12	» » » 6 »
S 155	6.5±1	0.71	1.48	3.39	1.74	1.88	» » » 5 min
S 161	10.4±1.3	0.76	1.77	3.89	1.32	1.37	» » » 10 »
G 4	22.5±2.25	4.4	2.37	3.08	1.39	1.26	» immediately
G 5	16.0±1.6	3.5	4.50	2.88	2.68	2.02	» »
G 9	6.3±0.63	1.6	2.20	3.69	2.04	1.80	
G 13	8.4±0.84	2.1	3.78	3.78	2.46	2.26	Died after 20 min
G 16	12.0±1.2	2.58	2.63	4.33	2.10	1.89	» immediately
G 17	10 ±1.0	2.36	3.29	4.16	1.97	1.51	» »
G 18	8.4±0.84	2.1	3.30	3.04	1.94	1.76	» »
G 23	6.5±0.65	1.04	3.09	3.44	1.59	1.26	» after 15 min
G 29	6.2±0.62	4.8	3.86	3.14	2.00	1.78	» » 2 hours 45 min

From a purely practical point of view it seems more valuable, therefore, to relate the degree of lesion to the more easily measurable quantities: distance from charge and weight of charge. The relation between these factors and fatal injury in rabbits caused by blast, is shown in the diagrams in fig. 23. In fig. 23 a the lines indicate maximum pressure and in fig. 23 b they indicate impulse. These diagrams are based on values obtained from a large number of experiments (ERICSSON and v. ZEIPPEL 1948) with the pressure- and impulse gauges used in the present study. The dotted line in the diagrams indicates the limit of mortal injuries to the rabbits in the

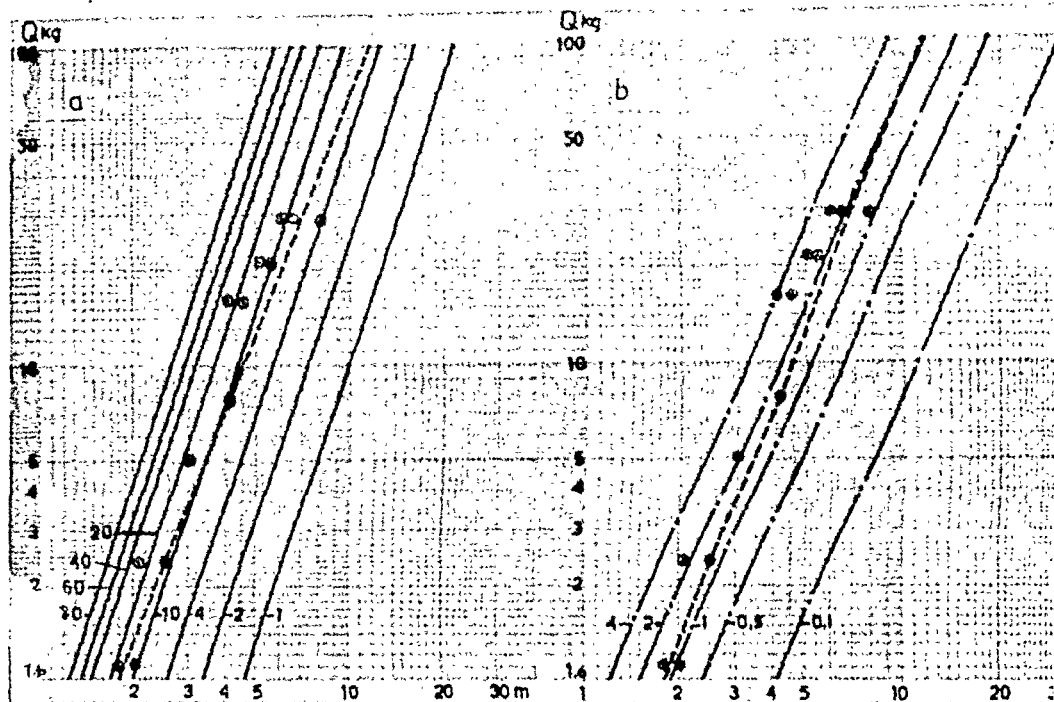


Fig. 23. Relation between weight of charge ( $Q$ ), distance from charge and mortal blast injuries in rabbits.  $\odot$  = animals killed by blast. — — — = limit for mortal injuries. Animals placed in points to the left of this line will receive mortal injuries. The parallel lines indicate: in *a*. max. blast pressures in  $\text{kgf}/\text{cm}^2$ , in *b*. impulses in  $\text{gf} \cdot \text{sec}/\text{cm}^2$ .

author's experiments. Animals placed to the left of this line have usually been deadly injured. As seen from the slope of the lines the genesis of deadly injuries to rabbits is proportional neither to maximum pressure nor to impulse. Both of these factors may cooperate.

#### The minimum survival distance.

Of great practical interest is the smallest distance from the charge at which the animal survives the detonation. A formula for "la distance minimum de survie" is found in the paper of BORIE (1947). This formula, which may be regarded as a special case of the scaling law mentioned on page 16, and which was originally calculated by a French explosives commission as an expression for "les distances dangereuses" (cf. BONNET 1918), is as follows:

$$R = C \cdot \sqrt[3]{Q}$$

where  $R$  = the distance from the charge in m, and  $Q$  = the weight of the charge in kg.  $C$  is a constant, dependent on the kind of explosive used. BONNET states that  $C$  for the explosives used by him, e.g. TNT, always was about 10.

Out of data for 37 rabbits, which, when exposed in the field, had died as a direct result of the detonation,  $C$  has been determined for TNT. It has been found, that  $C$  varies somewhat in relation to the weight of the charge. For very small charges ( $Q < 0.5$  kg) the value of  $C$  is about 2, but decreases with increasing weight of charge making it = 1.2 for  $Q = 100$  kg. From some experiments with a charge of 500 kg we get  $C = 1$ . The mean value for  $C$  is  $1.63 \pm 0.06$  ( $\sigma = 0.35$ ).

It is evident, therefore, that  $C$  in the formula mentioned above, is not constant.

As theoretical considerations seemed to prove that physiological effects should more likely vary with the cubic root of the weight of the charge the present author has calculated the following formula, instead of the one mentioned, for the shortest distance from the charge within which an animal can survive the detonation:

$$R = C \cdot \sqrt[3]{Q}$$

If  $C$  in this formula is determined as above, we get:

$$C = 1.90 \pm 0.04 \quad (\sigma = 0.26).$$

A certain dispersion exists even here, but  $C$  shows no tendency whatever to change in any special direction with different weights of charge.

Besides being dependent on the kind of explosive,  $C$  also appears to depend on the kind of animal used. A number of albino rats exposed to detonations at the same time as the rabbits, were considerably more sensitive than the rabbits and obtained in all respects more severe injuries than the rabbits. Most probably it is the size of the body that plays a part here, a fact pointed out earlier by ZUCKERMAN (1941), CLARK and WARD (1943), GREAVES *et coll.* (1943) and HORWATH and SHELLEY (1946), and others.

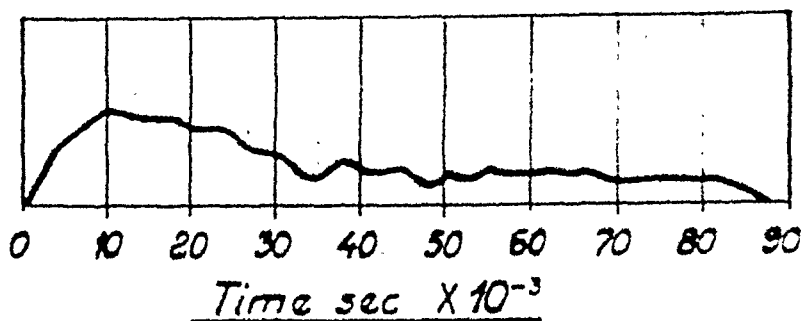
### Experiments in the detonation chamber.

The experiments in the detonation chamber show even clearer the importance of a combination between high pressure and great im-

[a



b



c

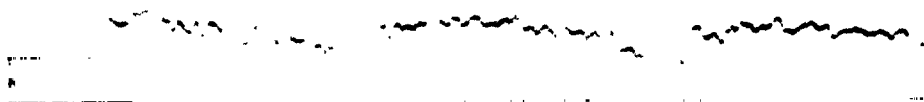


Fig. 24. The shock wave in the detonation chamber. a. Recorded shock diagram. b. The calibrated diagram of the same shock wave. c. Details of the first part of the shock wave showing oscillations and reflections from the walls of the chamber. This diagram is not recorded in the same blasting experiment as diagram a.

pulse in causing severe lesions. In spite of the fact that the impulses measured in the detonation chamber often have been ten times greater than those obtained with moderately great charges in the field, no severe injuries have appeared if the maximum pressure has been lower than  $4 \text{ kgf/cm}^2$  (see table 16).

In this connection the differences in nature of the blast wave in the field (fig. 1) and the blast wave in the detonation chamber may be pointed out. As seen from fig. 24 the latter consists of several reflected waves. In the field the pressure rises very rapidly and then falls again very rapidly. In the detonation chamber, on the contrary, the pressure rises considerably slower and lasts for a considerably longer time. These differences may be one of the reasons why a

TABLE 16. Quotients of lung injury of rabbits exposed to low max. pressures but very great impulses, in the detonation chamber.

(Max. pressure  $< 4$  kgf/cm<sup>2</sup>. Impulse  $> 20$  gf·sec/cm<sup>2</sup>.)

Rabbit	Max. pressure kgf/cm <sup>2</sup>	Impulse gf·sec/cm <sup>2</sup>	Quotients of lung injury			
			Haemorrhage (+ oedema)		Increase of lung volume (emphysema)	
			Left lung	Right lung	Left lung	Right lung
K 1	2.7	24.2	slight	moderate	—	—
K 2	3.0	24.2	0.91	0.91	1.0	1.05
K 3	2.8	30.0	1.66	1.20	1.18	1.09
K 4	3.28	25.2	0.93	0.95	1.03	0.99
K 5	3.49	24.3	1.22	1.26	0.93	0.89
K 6	3.69	25.3	1.38	1.39	1.19	0.99
K 7	3.49	23.7	slight	slight	—	—
K 8	3.42	25.7	»	»	—	—
K 9	3.22	25.7	0.97	1.04	0.95	1.02
K 10	3.3	24.0	0.88	0.87	0.91	0.94
K 11	2.49	22.5	0.85	0.88	1.10	1.05
K 12	2.7	24.5	0.67	0.82	1.01	1.00
K 14	3.34	41.0	1.06	1.08	0.98	0.90

greater impulse at a given maximum pressure is needed to produce lesions equivalent to those produced in field blasts. Another reason is probably the fact that the animal in the detonation chamber is not likely to be exposed to the effect of the suction wave. By being forced out of the chamber by the pendulum at the detonation it escapes, probably almost entirely, the effect caused by the negative phase of the blast wave. *This seems to prove, to a certain extent, that maximum lesion is caused through the combination of the positive and negative phases.* This problem, however, is not yet definitely solved. Earlier it was thought that lesion mainly depended on the effect caused by the suction wave (cf. LOGAN 1939, and others), a theory also supported by LATNER (1942) and by experiments with explosive decompression (EDELMAN et coll. 1946). According to ZUCKERMAN (1940, 1941), BERNAL (1941), and others, on the other hand, lesion is said to be mainly due to the positive pressure phase of the blast wave.

*In brief, it has been shown in this chapter that there exists a fairly*

good correlation between the maximum pressure and the impulse of a shock wave and the lung injuries caused by it. No such relation has been found in regard to abdominal injuries. Such injuries, especially intestinal haemorrhages, may be caused by shock waves of remarkably low maximum pressure and small impulse.

Injurious effects are caused only in the immediate vicinity of the detonating charge owing to the rapid decrease of the pressure- and impulse values. The minimum survival distance from a detonating charge can be obtained from the formula:  $R = C \cdot \sqrt[5]{Q}$ , the constant  $C$  being about 1.9 if the explosive is TNT and the animals used are rabbits.

## CHAPTER 12.

### The Effects of Blast Waves on Respiration.

As pointed out previously the pathological changes in the lungs in blast injury are fairly well described in a number of clinical and experimental papers (see e. g. COHEN and BISKIND 1946). On the other hand, the physiological reactions of the respiratory organs when affected by blast waves are still little analysed. Clinical investigations into changes in the function of the respiratory organs, for instance, type and rate of respiration etc. are noticeably sparse and incomplete, and in animal experiments recordings or even observations of respiration have been carried out only to a limited extent.

HADFIELD (1941) states that patients suffering from blast injury and treated by him showed an extreme expiratory dyspnoea. BARROW and RHOADS (1944) who have examined 200 cases of uncomplicated blast injury, found that on arrival to hospital the respiration of the injured was usually slow, shallow, weary. Pains in the chest in some cases seemed to modify the respiration, making it short and panting.

One of the reasons why respiration has hardly ever been recorded previously in blast experiments may be the lack of a recording method sufficiently simple but yet appropriate for the quite special conditions of such experiments.

The first to make extensive investigations into blast effects on certain physiological functions within the organism, was HOOKER (1924). He studied the respiration by graphic recording of the movements

of the chest with a pneumograph, but points out that the method was not suitable as the pneumograph could not be kept in the same position around thorax during the detonation. Owing to this, the respiration rate only could be obtained but not the amplitude and volume of the respiration. HOOKER found that the respiration in dogs exposed to the blast wave in front of a gun muzzle, was rapid and shallow. In an unanaesthetized dog the respiration was deep and strained but slow, and the respiratory muscles seemed to be insufficiently coordinated. The respiratory rate increased, and after one hour the animal had extreme air hunger. It died after about 40 hours.

An interesting investigation into blast effects on respiration has been made by KROHN, WHITTERIDGE and ZUCKERMAN (1942). They registered the action potentials in the diaphragm by means of insulated silver electrodes inserted into the xiphisternal slip of the diaphragm. The total electric activity in the vagus nerve was also recorded. Rabbits mainly were used for the experiments.

In a number of animals, less severely injured, there was an increase of the respiratory rate beginning within 15 or 20 seconds after the detonation and reaching up to ten times the initial frequency. In these cases some tonic discharge between the breaths was noted indicating that the diaphragm was not relaxed, and that the chest was in an inspiratory position.

In rabbits which survived blast pressures in the region of the  $P_{50}$  value (i. e. the maximum pressure at which 50 per cent of the animals immediately died), a brief apnoe set in followed by a slow, irregular, jerky breathing which was later succeeded by a rapid, regular and shallow breathing. The period of apnoe lasted 5 to 20 seconds. During this there was a strong centripetal discharge in vagus, and a noticeably strong activity was maintained in the diaphragm suggesting vigorous attempts at inspiration. The respiration rate reached maximum after approximately half an hour, after which it gradually decreased.

Severely injured animals died after a few minutes with slow, jerky breathing. It was unusual that the animals died without making any attempts at inspiration after the explosion.

In cases where lung oedema developed the respiration became slower and deeper. The blood-pressure dropped, and signs of asphyxia set in.

CLARK and WARD (1945) who have studied on a small scale the

effect on the organism of shock waves in water, found that after exposure to a shock wave with fatal effect an apnoe instantly set in which lasted from a few seconds to more than one minute.

The statements in the literature concerning the effect of blast waves on respiration are, however, incomplete. Thus, for example, no investigations have been made as to the differences in the effects caused by blast waves with different maximum pressures and impulses. The question whether changes in respiration would be the direct cause of death in blast injury has not been sufficiently analysed.

### Own investigations.

Respiration has been recorded by the thermoelectric recording method described on page 31. In most cases electrocardiograms have been taken simultaneously. The recording has begun 5 or 10 seconds before the detonation *and has continued without any interruption till 30 or 60 seconds after the detonation.* After that recordings have been taken at different times up to 24 hours after the explosion. *The chief object, however, has been to study the changes appearing immediately after the detonation.* These experiments comprise 42 animals, *all anaesthetized.*

Before describing the results of the blasting experiments it seems appropriate to touch upon the features of the respiration curve obtained from normal anaesthetized rabbits by the recording method used here.

### Respiration of normal rabbits.

The respiration rate varies quite considerably in the animals used. The number of breaths per minute varies between 22 and 84 with an average of 45 per minute in the 42 animals. The reasons for these great variations are probably several, of which may be mentioned individual differences and different depths of anaesthesia. In the individual animal the respiration is quite regular both as to rate and amplitude.

The shape of the respiration cycle also varies in the different animals. The inspiratory phase is generally briefer than the expiratory phase. The mean relation between the duration of inspiratory and that of expiratory phase is 1: 1.4. In rapid breathing the two phases are often equally long. In some cases the inspiration is even longer.



In slow respiration there is occasionally a neutral interval of up to 0.5 seconds between the two respiratory phases.

It may be pointed out here that no absolute values as regards the amplitude and the ventilation can be obtained by the method used as the amplitude of the oscillogram, to a certain extent, depends on the temperature of the surrounding air.

### Respiration of blasted animals.

At the moment of the detonation a more or less marked distortion of the respiratory curve occurs, owing to the heat impulse from the blast. This is of very brief duration, however, and even in the most violent blasts it has thus been possible to determine the effect on the respiration already after a period of less than two seconds after the detonation. Owing to the distortion of the curve, and the relatively great speed of the film (40 mm/sec), it is possible to determine with the utmost exactitude the respiratory position of the animal when struck by the blast wave.

The material has been divided into four groups according to the magnitude of the maximum pressure and impulse of the blast wave. The limits between the groups have been chosen at approximately those pressure and impulse values at which severe lung lesions occur. The four groups are:

1. *Low maximum pressures ( $< 4 \text{ kgf/cm}^2$ ) and small impulses ( $< 0.5 \text{ gf} \cdot \text{sec/cm}^2$ ).*
2. *High maximum pressures ( $> 4 \text{ kgf/cm}^2$ ) and small impulses ( $< 0.5 \text{ gf} \cdot \text{sec/cm}^2$ ).*
3. *Low maximum pressures ( $< 4 \text{ kgf/cm}^2$ ) and great impulses ( $> 0.5 \text{ gf} \cdot \text{sec/cm}^2$ ).*
4. *High maximum pressures ( $> 4 \text{ kgf/cm}^2$ ) and great impulses ( $> 0.5 \text{ gf} \cdot \text{sec/cm}^2$ ).*

#### 1. The effects of low maximum pressures and small impulses.

The animals belonging to this group have been exposed to detonations of small charges (weight of charge  $230 \pm 5 \text{ g}$ ) in the field ("group type A").

Table 17 a gives the pressure and impulse values and respiratory rate as well as the quotients of lung lesion of the animals belonging

TABLE 17 a. Max. pressure and impulse values, distance from charge, respiration rate and quotients of lung injury of rabbits exposed to shock waves with low max. pressure and small impulse.

Weight of charge in all experiments 0.23 kg.

Rabbit	Distance from charge m	Max. pressure kgf/cm <sup>2</sup>	Impulse gf · sec/cm <sup>2</sup>	Respiration rate			Quotients of lung injury			
				Before detonation	After <sup>1</sup> detonation	Per cent increase	Haemorrhage (+ oedema)		Increase of lung volume (emphysema)	
							Left lung	Right lung	Left lung	Right lung
S 166	3.50	0.48	<0.12	50	50	0	1.03	1.01	0.94	0.91
S 167	3.0	0.53	0.18	44	47 (2 sec)	7	1.05	1.05	1.02	1.01
S 168	2.0	1.44	0.12	40	33 (30 sec)	—18	0.95	0.93	1.01	1.06
S 174	1.85	1.87	<0.06	30	72 (20 min)	140	0.81	0.88	1.02	0.99
S 173	1.70	2.39	0.04	53	83 (40 min)	57	1.18	1.15	1.11	0.93
S 171	1.40	3.43	0.09	33	136 (15 sec)	312	1.17	1.50	1.05	1.16
S 169	1.50	3.74	0.18	28	43 (10 sec)	54	0.94	0.97	1.24	0.84

TABLE 17 b. Mean value of respiration rate during the first few minutes after the detonation, of rabbits exposed to shock waves with low max. pressure and small impulse.

	Before detonation	Time after detonation				
		Immediately	15 sec	30 sec	2 min	3 min
<i>n</i>	7	6	7	5	5	3
<i>M</i>	39.7	63.3	57.4	54.4	48.6	55.0
<i>ε</i> ( <i>M</i> )	3.7	11.5	13.5	11.7	8.0	10.1
<i>σ</i>	9.8	28.2	35.5	26.3	17.9	17.4

to this group. The animals have been grouped according to increasing pressure values.

The mean value of the respiration rate at different times after the detonation is shown in figure 25 (curve I). The rate increase varies quite considerably in different animals, why the standard deviation becomes fairly great (see table 17 b).

Very small max. pressures and impulses have no effect on the respiration (e. g. rabbit S 166, table 17 a). At somewhat higher values

<sup>1</sup> The time, given within brackets, is the time after the detonation at which the greatest respiration rate found here has been recorded.

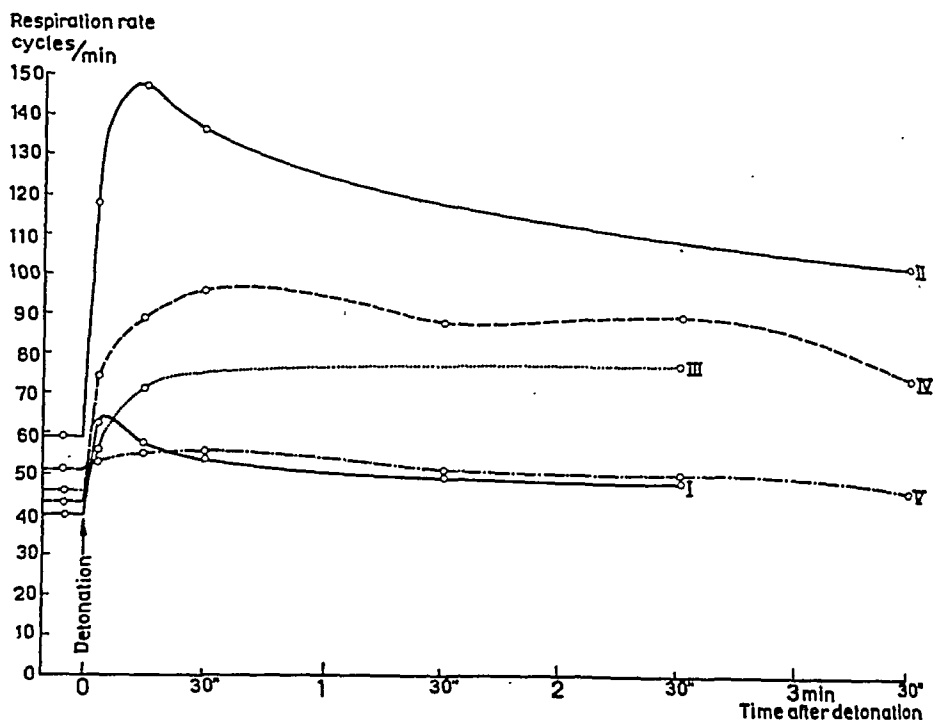


Fig. 25. Mean value of respiration rate during the first minutes after detonation, of rabbits exposed to shock waves with: I. low max. pressure and small impulse. II. high max. pressure and small impulse. III. low max. pressure and great impulse. IV. high max. pressure and great impulse. V. *Bilaterally vagotomized* animals exposed to high max. pressure and great impulse.

(0.5—1 kgf/cm<sup>2</sup>) a slight increase of the respiration rate immediately after the detonation is the only result. This increase is of short duration. In animal S 167 for instance, which had been exposed to a maximum pressure of 0.53 kgf/cm<sup>2</sup> and an impulse of 0.18 gf · sec/cm<sup>2</sup> the respiration rate rose from 44 before to 47 breaths per minute after the explosion. Already a few minutes later, however, the rate was again the same as before the detonation.

In some cases there was a period just after the detonation with a few short, irregular breaths. In one of the animals (S 168) these were followed by a period of apnoe lasting about 8 seconds and which was succeeded by a few unequal, slow breaths. Not until about 25 seconds after the detonation the respiration was again regular. The rate then, however, was somewhat lower than before the detonation.

The greatest increase of the respiration rate is found in animal S 171 where it is 115 per minute already one or two seconds after the ex-

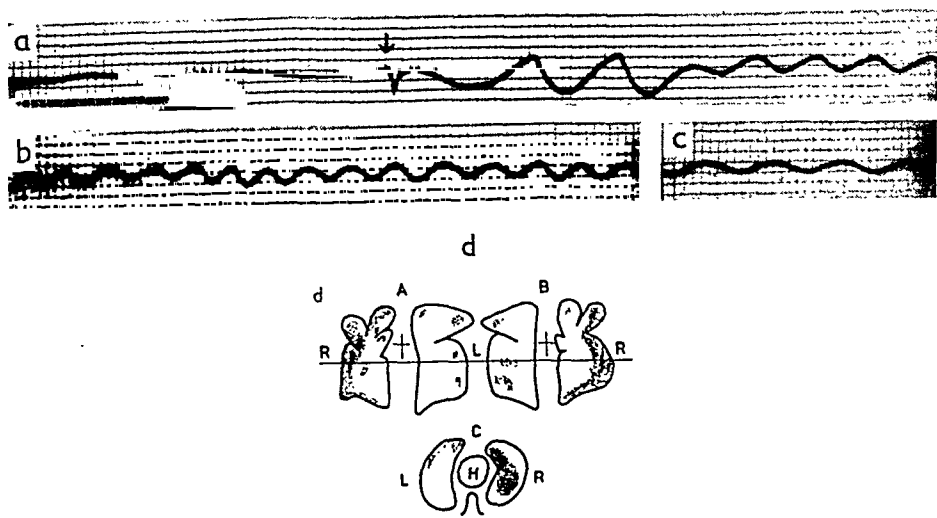


Fig. 26. Respiration. Rabbit S 171, exposed to a max. blast pressure of 3.43  $\text{kgf/cm}^2$  and an impulse of 0.09  $\text{gf} \cdot \text{sec/cm}^2$ . a. Before and until 7 sec after the detonation which occurred just at the arrow. b. 22—30 sec and c. 2 min after the detonation. Time marking 0.1 sec. d. Schematic figure of the lungs with haemorrhages (A = ventral and B = dorsal surface. C = cross-section. R = right and L = left lung. H = heart).

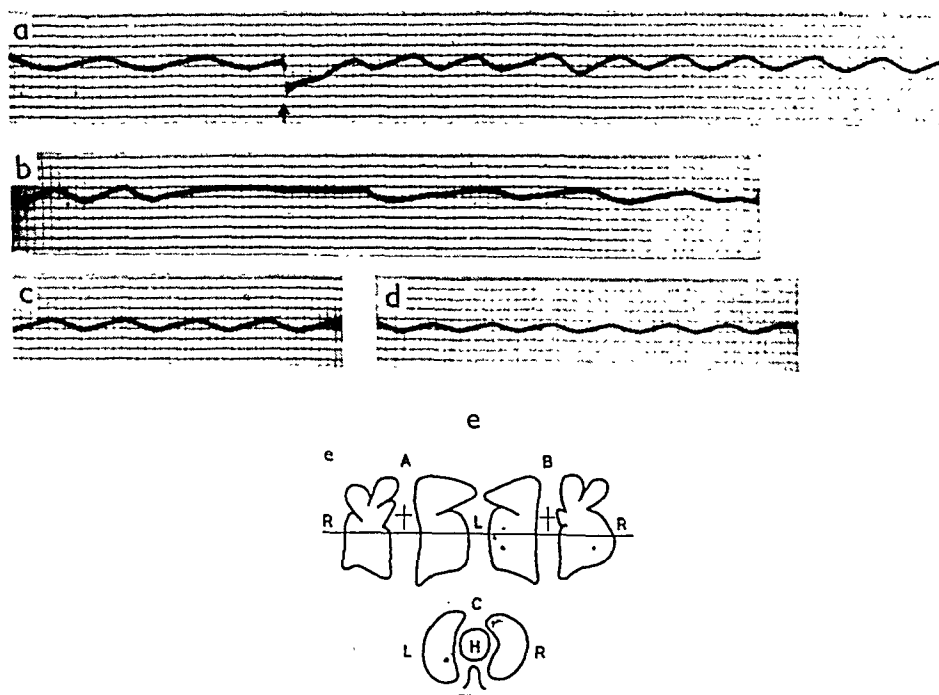


Fig. 27. Respiration. Rabbit S 173, exposed to a max. blast pressure of 2.4  $\text{kgf/cm}^2$  and an impulse of 0.04  $\text{gf} \cdot \text{sec/cm}^2$ . a. Before and until 8 sec after the detonation (detonation at arrow). b. 9—18 sec. c. 4.5 and d. 40 min after the detonation. Time marking 0.1 sec. e. Schematic figure of the lungs with haemorrhages (explanation of the letters see fig. 26).

plosion (see fig. 26). Five minutes after, however, it has again dropped to 63 cycles per minute. In other cases the respiration increases slowly not reaching maximum value until 30 minutes have passed (fig. 27), after which it again slowly decreases. *The mean value of the maximum increase of the respiration rate observed within this group, is 79 per cent.*

No other obvious changes of the respiration curve is seen, except in rabbit S 171. The respiration of this animal before the detonation was slow and had a very small amplitude. Immediately after the detonation there were two breaths with an amplitude approximately six times greater than the initial amplitude. After this the respiration was regular but with much greater frequency and amplitude than before the detonation.

The amplitude immediately after the detonation is generally greater than before the detonation, but it decreases with increasing frequency and becomes smaller than before the explosion.

It is clear from table 17 a that significant lung haemorrhages exist only in animal S 171, while the lungs in S 166 and S 174 are quite uninjured. In the other animals in this group only slight haemorrhages are found.

## 2. The effects of high pressures and small impulses.

The animals in this group have also been exposed to detonations of small charges of TNT in the field. Owing to the difficulty in obtaining high pressures and small impulses, the group consists of only 3 animals. Data for these are found in table 18. Rabbit S 175 has been included here in spite of the impulse being somewhat greater than 0.5.

Respiration in all three animals continues after the detonation without any period of apnoe. It is rapid, shallow and has a very small amplitude. This concerns animal S 170 particularly, which has been exposed to a maximum pressure of  $11.2 \text{ kgf/cm}^2$  and an impulse of  $0.48 \text{ gf} \cdot \text{sec/cm}^2$  (fig. 28). After a period of distortion lasting 1.7 seconds immediately after the explosion, six breaths follow here during 2.3 seconds (160 per minute), after which follows a period of a good 30 seconds with a frequency of 210 breaths per minute and an amplitude of only one tenth of the original. Four minutes after the detonation the frequency is still approximately 120/min. The ampli-

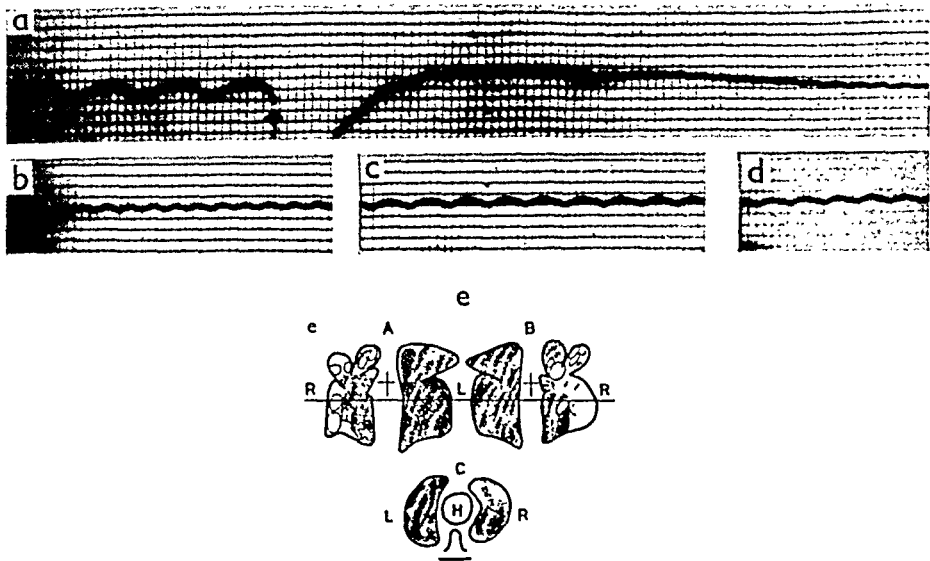


Fig. 28. Respiration. Rabbit S 170, exposed to a max. blast pressure of 11.2 kgf/cm<sup>2</sup> and an impulse of 0.48 gf · sec/cm<sup>2</sup>. a. Before and until 8 sec after the detonation, which occurred just at the arrow. b. 20—24 sec, c. 1.5 and d. 2.5 min after the detonation. Time marking 0.1 sec. e. Schematic figure of the lungs with haemorrhages and bullous emphysema (explanation of the letters see fig. 26).

tude has increased somewhat. The percental maximum increase of the respiration rate is of the same magnitude in all three animals, namely 168 per cent in S 175, 162 per cent in S 172 and 196 per cent in S 170, *with a mean value of 175 per cent*. The mean value of the respiration rate is shown in fig. 25 (curve II).

The frequency decreases and the amplitude increases only quite slowly and it will be several hours before the values are the same as before the detonation. No important changes of the shape of the respiratory curve appears, with the exception of a certain change in the relation between the duration of the inspiration and that of the expiration. Thus, the expiratory phase, from having been briefer than the inspiratory phase in some animals before the detonation, becomes extended, and finally is as long as, or longer than, the inspiratory phase.

Rabbits S 172 and S 175 have obtained only slight to moderate haemorrhages, while animal S 170, on the other hand, as shown in table 18, has very severe lung injuries with haemorrhages throughout the left lung, and an abundance of blood, blood-tinged frothy fluid and clots in the upper respiratory passages. The animal, however, did not

TABLE 18. Max. pressure and impulse values, distance from charge, respiration rate and quotients of lung injury of rabbits exposed to shock waves with high max. pressure and small impulse.

Weight of charge in all experiments 0.23 kg.

Rabbit	Distance from charge m	Max. pressure kgf/cm <sup>2</sup>	Impulse gf · sec/cm <sup>2</sup>	Respiration rate			Quotients of lung injury			
				Before detonation	After detonation <sup>1</sup>	Per cent increase	Haemorrhage (+ oedema)		Increase of lung volume (emphysema)	
							Left lung	Right lung	Left lung	Right lung
5	1.15	8.6	0.66	44	118 (33 min)	168	1.10	1.07	0.95	0.9
2	1.25	8.84	0.43	63	165 (5 min)	162	1.02	1.23	0.98	0.9
0	1.0	11.2	0.48	71	210 (5 sec)	196	5.82 <sup>2</sup>	1.65 <sup>2</sup>	1.48	1.3

TABLE 19 a. Max. pressure and impulse values, respiration rate and quotients of lung injury of rabbits exposed in the detonation chamber to shock waves with low max. pressure and very great impulse.

Rabbit	Max. pressure kgf/cm <sup>2</sup>	Impulse gf · sec/cm <sup>2</sup>	Before detonation	Respiration rate		Quotients of lung injury			
				After detonation <sup>3</sup>	Per cent increase	Haemorrhage (+ oedema)		Increase of lung volume (emphysema)	
						Left lung	Right lung	Left lung	Right lung
K 11	2.49	22.5	32	39 (30 sec)	22	0.85	0.88	1.10	1.0
K 7	3.49	23.7	50	70 (1,5 min)	40	slight	slight	—	—
K 10	3.30	24	34	55 (2 min)	62	0.88	0.87	0.91	0.9
K 1	2.70	24.2	38	56 (15 sec)	47	slight	moderate	—	—
K 2	3.0	24.2	38	86 (15 sec)	126	0.91	0.91	1.0	1.0
K 5	3.49	24.3	60	100 (20 sec)	67	1.22	1.26	0.93	0.8
K 12	2.70	24.5	47	78 (15 sec)	66	0.67	0.82	1.01	1.0
K 4	3.28	25.2	57	72 (1,5 min)	26	0.93	0.95	1.03	0.9
K 6	3.69	25.3	77	100 (15 sec)	30	1.38	1.39	1.19	0.9
K 9	3.22	25.7	38	40 (15 sec)	5	0.97	1.04	0.95	1.0
K 3	2.80	30	39	104 (30 sec)	167	1.66	1.20	1.18	1.0
K 14	3.34	41	38	92 (30 sec)	142	1.06	1.08	0.98	0.9

<sup>1</sup> The time, given within brackets, is the time after the detonation at which the greatest respiration rate found here has been recorded.

<sup>2</sup> At detonation this rabbit had its left side turned against the charge.

<sup>3</sup> The time, given within brackets, is the time after the detonation at which the greatest respiration rate found here has been recorded.

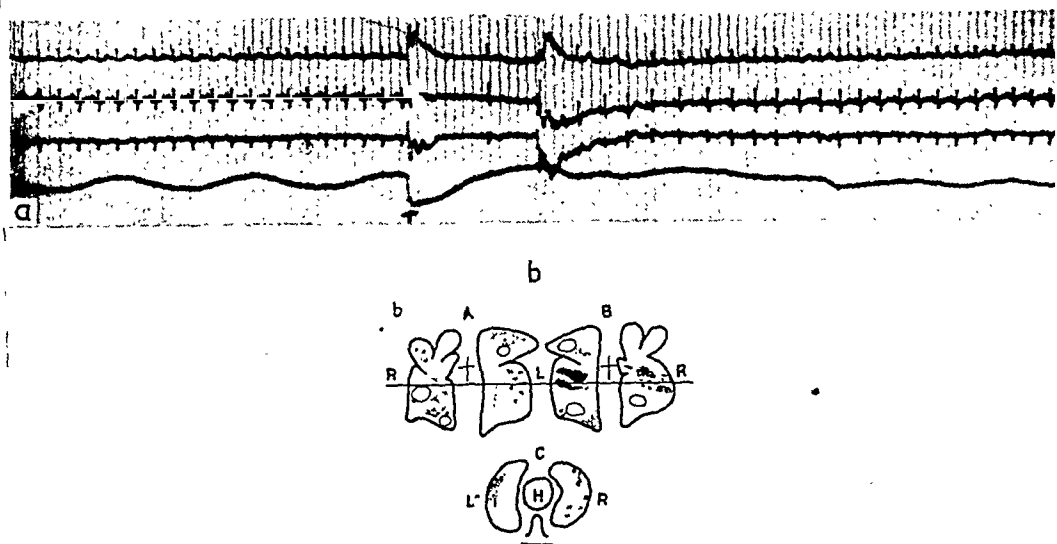


Fig. 29. Rabbit K 14. Electrocardiogram and respiration. The animal has been exposed to a max. blast pressure of  $3.34 \text{ kgf/cm}^2$  and an impulse of  $41 \text{ gf} \cdot \text{sec/cm}^2$ . *a.* Before and until 7 sec after the detonation (at arrow). *b.* Schematic figure of the lungs with haemorrhages and bullous emphysema.

bled from the nose. None of these animals have died from the injuries.

### 3. The effects of low maximum pressures and great impulses.

All experiments in this group have been carried out in the detonation chamber. The weights of charge have varied between 4 and 6 g. In the experiment with rabbit K 1, a charge of 10 g of *nitrolite* (an ammonium nitrate explosive), has been used.

Data concerning these animals are found in table 19 a. The animals have been grouped according to increasing impulse values.

TABLE 19 b. Mean value of respiration rate during the first few minutes after the detonation, of rabbits exposed in the detonation chamber to shock waves with low max. pressure and very great impulse.

	Before detonation	Time after detonation			
		Immediately	15 sec	30 sec	2 min
<i>n</i>	12	12	12	8	7
<i>M</i>	45.7	56.1	70.5	72.3	78.1
<i>ε (M)</i>	3.8	3.9	6.0	8.8	7.4
<i>σ</i>	13.3	13.6	20.9	25.0	19.7



TABLE 20 a. Max. pressure and impulse values, respiration rate and quotients of lung injury of rabbits exposed in the detonation chamber to shock waves with high max. pressure and great impulse.

Rabbit	Max. pressure kgf/cm <sup>2</sup>	Impulse gf·sec/cm <sup>2</sup>	Respiration rate		Per cent increase	Quotients of lung injury				Comments
			Before detonation	After detonation <sup>1</sup>		Haemorrhage (+ oedema)		Increase of lung volume (emphysema.)		
						Left lung	Right lung	Left lung	Right lung	
K 15	4.48	45.2	53	150 (15 sec)	183	0.87	0.85	1.02	1.08	Died after 20 min
K 43	5.1	5.4	44	110 (30 »)	150	1.11	1.06	1.00	0.93	
K 20	5.35	9.8	39	120 (15 »)	208	1.06	0.87	0.88	0.96	
K 35	6.7	7.6	50	105 ( 1 min)	110	1.26	1.31	1.08	1.17	
K 25	8.2	15.6	32	96 ( 1 » )	200	1.46	1.30	1.24	1.14	
K 21	8.46	22.1	41	106 ( 3 » )	158	—	—	—	—	Died within a few min
K 33	9.2	12.8	34	125 (30 sec)	268	moderate	severe	—	—	
K 39	9.7	11.3	41	110 (30 »)	168	1.21	1.84	0.96	1.09	
K 22	9.85	24.4	33	80 (10 »)	142	1.58	0.96	1.11	1.25	
K 23	11.6	22.6	22	80 (30 »)	264	2.74	1.58	1.66	0.99	
K 32	11.9	20.5	49	86 (30 »)	76	1.64	2.06	0.98	0.98	Died after 10 min
K 27	11.9	20.8	27	56 (15 »)	107	1.33	1.74	1.12	1.25	
K 29	12.5	25.0	27	30 ( 2 min)	11	1.73	1.99	1.12	1.14	
K 36	12.5	11.9	56	178 (30 sec)	218	1.02	1.20	1.17	1.27	
K 24	12.6	22.1	84	115 ( 3 min)	37	3.79	1.79	1.71	1.22	
K-31	13.3	23.9	67	80 <sup>2</sup> (immed.)	19	1.20	1.36	1.01	1.01	Died after some min Died after 20 min
K 40	13.3	17.0	32	52 (30 sec)	63	2.77	3.16	1.53	1.65	
K 37	14.8	22.2	56	158 ( 4 min)	182	2.08	3.15	1.44	2.25	
K 16	16.4	35.4	32	73 (15 sec)	128	3.17	2.48	1.69	1.84	
K 19	16.6	24.6	45	90 (immed.)	100	3.86	3.05	2.05	1.68	

<sup>1</sup> The time, given within brackets, is the time after the detonation at which the greatest respiration rate found here has been recorded.

<sup>2</sup> The value is uncertain.

After a slight distortion of the curve the respiration can be observed in these animals within one second after the detonation. The respiration continues immediately without any apnoe, usually with a deep inspiration, which in some cases lasts several seconds. The respiration is usually regular all the time after the explosion, only in a few cases a somewhat irregular breathing is noticed during the first 10 or 20 seconds (see fig. 29).

The respiration rate increases almost immediately after the detonation (see fig. 25, curve III). *This increase varies considerably in different animals, but in most cases it is only quite moderate amounting to an average of 32 per cent immediately after the explosion (limit values: 5 and 97 per cent).* In one case (K 5) however there is a decrease of about 5 per cent immediately after the detonation. During the next few minutes a further increase is seen in most cases, and after about 3 minutes it has reached an average increase of 62 per cent (limit values: 5 and 167 per cent). *The average maximum increase recorded is now 67 per cent.*

The greatest increase of the respiration rate, namely from 39 breaths per minute before to 104 per minute at approximately 3 minutes after the detonation (an increase of 167 per cent), is seen in rabbit K 3 which also has the most extensive lung haemorrhages. All other animals have only insignificant or slight lung lesions. Animals K 2 and K 4 were almost entirely uninjured. All animals belonging to this group have survived.

#### 4. The effects of high maximum pressures and great impulses.

This group consists of 20 animals, all exposed in the detonation chamber.

Pressure- and impulse values and quotients of lung lesions of these animals are found in table 20 a.

The mean value of the respiratory rate is shown in table 20 b and fig. 25, curve IV. The dispersion is considerable, as shown in the table.

Two of the animals in this group have obtained such severe injuries that they have died almost immediately without the respiration having got properly started again after the explosion. In one of these (K 19) there is possibly some respiration but it is so irregular, however, and has such a small amplitude that it is almost impossible to estimate. The frequency seems to be about 90 breaths per minute

Table 20 b. Mean value of respiration rate during the first few minutes after the detonation, of rabbits exposed in the detonation chamber to shock waves with high max. pressure and great impulse.

	Before detona- tion	Time after detonation							
		Im- mediately	15 sec	30 sec	1 min	2 min	3 min	4 min	5 min
<i>n</i>	20	15	14	10	9	8	7	5	4
<i>M</i>	43.0	74.6	89.1	95.6	87.7	89.8	75.0	108.6	80.0
$\varepsilon(M)$	3.3	7.4	10.3	11.9	7.4	16.2	13.1	21.2	13.1
$\sigma$	17.7	27.8	38.5	37.6	22.2	45.8	34.8	47.5	26.1

(initial value: 45 per minute). The respiration has ceased already within one minute after the detonation.

In six of the animals which have survived the detonation, a respiratory stand-still in an inspiratory position follows immediately after the detonation. Its duration varies from 2.5 to more than 60 sec. with an average of 22 sec. When respiration starts again it is often extremely irregular. In some cases, the apnoe is interrupted by a few irregular breaths followed by another, shorter period of respiratory stand-still.

In all animals which have survived the detonation, a more or less pronounced increase of the respiration rate begins sooner or later. Maximum is generally reached already within one or two minutes after the detonation. Only in three animals has maximum been reached later, namely, in rabbits K 21 and K 24 after about three minutes, and in K 37 not until about ten minutes after the detonation. *The average maximum increase recorded is 140 per cent.* The respiration is usually extremely irregular both in frequency and amplitude.

The respiration rate decreases again gradually but in most cases it is for several hours considerably higher than before the detonation, and the animals often show quite a pronounced dyspnoea. During the first hours they are more or less cyanotic. In one animal, S 176, exposed to a maximum pressure of 32 kgf/cm<sup>2</sup> and an impulse of 3.2 gf·sec/cm<sup>2</sup> (charge weight 230 g and distance from charge 0.90 m), the respiration rate was 43 breaths per minute before the detonation, and even 24 hours after the explosion, it was 73 per min. The animal, however, had then begun to wake up again. Only in one of the surviving animals (K 43) the frequency had dropped already

within 5 minutes to a value (30 per min) which was lower than before the detonation (44 per minute).

As seen from table 20 *a the greatest increase of the respiration rate generally occurred in animals exposed to the lowest pressure values.* Thus the mean value of the percental increase is for the 10 animals which have been exposed to the lowest pressures,  $185.1 \pm 16.2$  per cent, while for the 10 animals which have obtained the highest pressures, it is only  $94.1 \pm 21.5$  per cent. This difference is statistically highly probable ( $0.01 > P > 0.001$ ).

In cases where the frequency does not reach maximum immediately after the detonation, the increase is usually gradual. In rabbit K 43, however, 10 seconds after the detonation, the respiration rate changed suddenly in one single breath from 73 to 94 breaths per minute.

The amplitude decreases after the detonation in all animals. When the frequency is at its greatest the amplitude is generally only one fourth to one fifth of what it was before the explosion. When the frequency then again gradually decreases, a corresponding increase of the amplitude is usually seen. In spite of this *there is a considerable reduction of the total pulmonary ventilation.*

In several animals of this group, for instance rabbit K 19, very strong respiratory movements were seen during several minutes immediately after the detonation, and the auxiliary respiratory muscles were employed in the highest possible degree. *In spite of these very strong respiratory movements the ventilation was insignificant, and in some cases hardly any respiration could be observed on the pneumogram.*

Some experiments have been made in which the animals have been exposed in the detonation chamber, *enclosed totally except for their heads in a steel cylinder.* These animals, even if exposed to max. pressures and impulses great enough to cause instantaneous death, have not obtained any pulmonary haemorrhages whatsoever but only slight diffuse emphysema. *In these animals respiration is not, or only little, affected.* Only a slight increase may be seen for some seconds after the detonation.

### **The effects of shock waves on the respiration of vagotomized animals.**

Blast experiments on animals whose vagal reflex mechanism has been interrupted, are extremely few.

TABLE 21 a. Max. pressure and impulse values, respiration rate and quotients of lung injury of rabbits which after *bilateral vagotomy* have been exposed in the detonation chamber.

Rabbit	Max. pressure kgf/cm <sup>2</sup>	Impulse gf · sec/cm <sup>2</sup>	Respiration rate			Quotients of lung injury				Comments
			Before detonation	After detonation <sup>1</sup>	Per cent increase	Haemorrhage (+ oedema)		Increase of lung volume (emphysema)		
						Left lung	Right lung	Left lung	Right lung	
K 49	3.8	3.2	48	56 (immed.)	17	1.18	1.14	1.05	1.07	Died after 20 min
K 50	9.8	11.4	41	56 (30 sec)	37	1.42	1.51	1.10	1.16	Died after 15 min
K 55	10.2	12.7	32	40 (30 »)	25	1.71	2.33	1.42	1.85	
K 48	11.0	12.7	37	46 (30 »)	24	1.19	1.97	1.09	1.78	
K 51	11.2	13.0	86	91 (15 »)	6	1.11	1.73	1.18	1.08	
K 46	11.4	16.2	39	34 (immed.)	—13	1.51	2.78	1.42	1.93	Died after some min
K 52	12.3	15.1	69	69 (15 sec)	0	1.43	1.99	1.61	2.72	Died after 5 min
K 54	12.8	15.1	62	63 (immed.)	2	1.13	1.38	1.04	1.13	
K 45	14.0	18.1	48	62 (15 sec)	29	1.50	2.97	1.45	1.95	Died after 5 min
K 44	14	18.1	42	30 (15 »)	—29	2.34	3.36	1.38	2.17	Died after some min
K 38	14.2	24.3	29	45 (30 »)	55	1.06	1.22	0.94	1.01	Died after about 10 min
K 17	14.7	31.0	35	32 (immed.)	—9	3.45	3.09	2.22	1.99	Died after some min
K 41	19.2	26.0	22	17 ( » )	—23	1.86	2.00	1.30	1.26	Died after some min

<sup>1</sup> The time, given within brackets, is the time after the detonation at which the greatest respiration rate found here has been recorded.

TABLE 21 b. Mean value of respiration rate during the first few minutes after detonation of rabbits which after *bilateral vagotomy* have been exposed in the detonation chamber.

	Before detonation	Time after detonation					
		Im- mediately	15 sec	30 sec	1 min	2 min	3 min
<i>n</i>	9	9	9	9	6	5	5
<i>M</i>	50.2	53.6	55.6	54.6	49.7	50.0	47.2
$\varepsilon(M)$	6.3	4.3	5.6	4.4	7.5	10.2	9.9
$\sigma$	18.7	12.8	16.9	13.2	22.0	22.7	22.1

KROHN, WHITTERIDGE and ZUCKERMAN (1942) in one experiment on a rabbit found that bilateral vagotomy stopped the rapid breathing caused by the detonation, and that it was replaced by the ordinary deep vagal respiration. The animal showed no signs of cyanosis. Also CLARK and WARD (1943) observed that vagotomy reduced the respiratory rate in animals injured by blast. The results are of great interest, and as these experiments seem to give valuable information as to essential features in the physiological effects of the blast wave, the present author has carried out a number of such experiments.

The material consists of 15 animals exposed in the detonation chamber to great impulse and as a rule high maximum pressure. Before the experiment the vagus nerve was cut on both sides of the neck. A tracheal cannula was inserted in some of the animals. The animals were exposed to the detonation approximately half an hour after the vagotomy.

Two of the animals were in a bad condition already before the detonation and had slow, irregular breathing. They died immediately after the explosion. These animals have not been included in the results.

Several of the vagotomized animals were killed outright by the detonation without the respiration having got properly started again.

In the other animals there was only a very slight increase of the respiration rate compared to that in the untreated animals (see table 21 a).

*The mean value of the maximum percental increase of the respiration rate is only 11 per cent.* In some of the animals, which have died some minutes after the detonation, the respiration rate immediately after

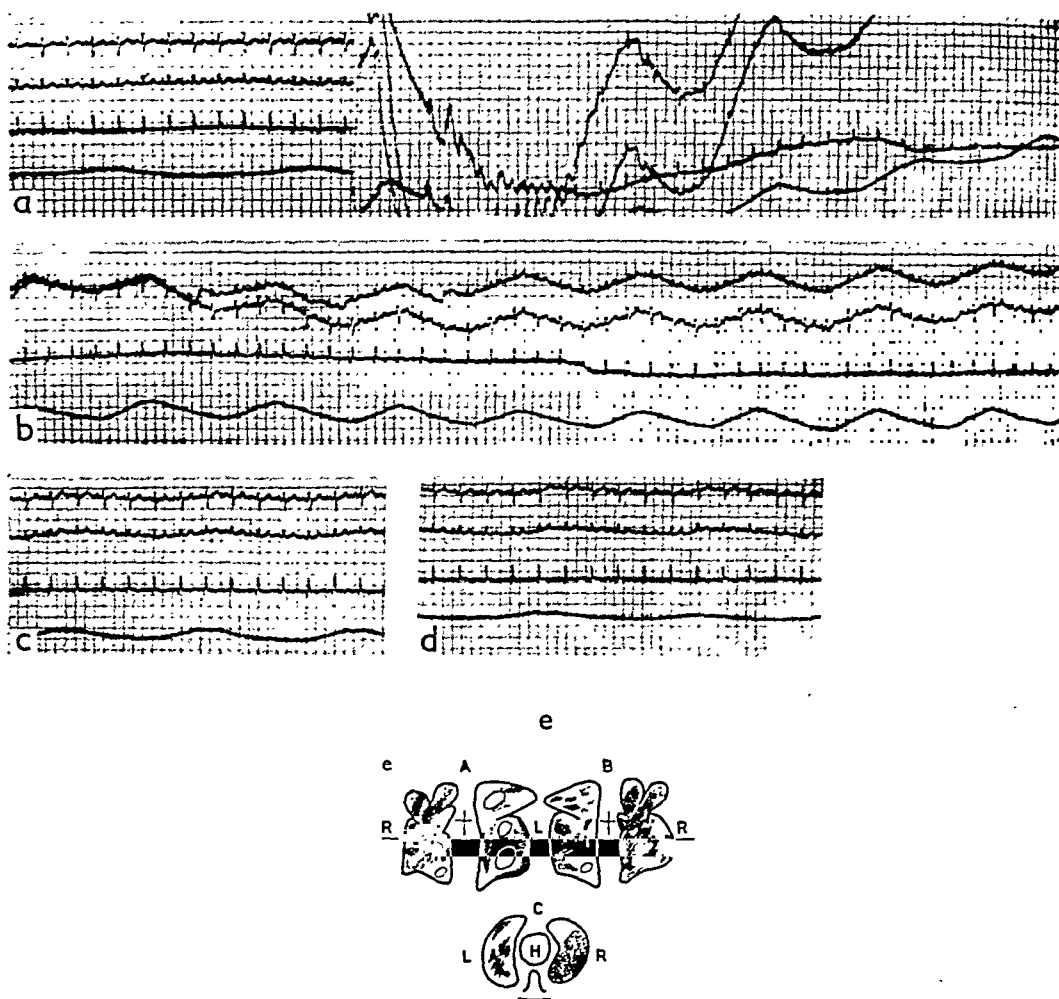


Fig. 30. Electrocardiogram and respiration of rabbit K 38, which after *bilateral vagotomy* has been exposed to a max. blast pressure of  $14.2 \text{ kgf/cm}^2$  and an impulse of  $24.3 \text{ gf} \cdot \text{sec/cm}^2$ . *a.* Before and until 7.5 sec after the detonation (detonation at the severe distortion of the curves). *b.* 20—32 sec, *c.* 6.5 and *d.* 11.5 min after the detonation. Time marking 0.1 sec. *e.* Schematic figure of the lungs with haemorrhages and bullous emphysema (explanation of the letters see fig. 26).

this has been lower than the initial value, which has never been reached again.

The mean value of the respiration rate at different times after the detonation is shown in fig. 25 (curve V).

The respiration during the first 10 or 20 seconds after the detonation is irregular both in frequency and amplitude, and even the shape of the respiratory cycles often shows irregularities, an occurrence common even in unvagotomized animals. In these a more or less marked de-

crease of the amplitude was a regular occurrence. *In the vagotomized animals, however, the amplitude increases after the detonation and is in some cases almost twice as great after the detonation as before* (see fig. 30).

### Arterial oxygen saturation.

As an indication of the oxygenation of the blood becoming more difficult, cyanosis sets in within a short while after the detonation. Especially in the severely injured, unanaesthetized animals the cyanosis may be very pronounced.

It was of great interest therefore, to determine the arterial oxygen saturation. This has been done in a few animals by using the bloodless method, described on page 36.

All animals examined 5 or 10 minutes after the detonation have shown a reduction of the arterial oxygen saturation. The reduction, however, is only moderate in most cases, and varies in the different animals from 4 to 26 per cent.

Determinations made 3 or 4 hours later showed that the initial value had again been reached. In anaesthetized animals cyanosis had generally disappeared a few hours after the detonation.

No determinations of arterial oxygen saturation has, owing to technical reasons, been made in unanaesthetized rabbits, but it may be pointed out here, that cyanosis in these animals usually was more pronounced and lasted for several hours.

### Discussion.

The present investigations confirm the observations made previously by KROHN *et coll.* (1942), namely, that the respiration is conspicuously changed in animals exposed to the effects of the pressure- and impulse field around a detonating charge.

In animals which have obtained severe lung lesions due to blast there is usually a marked increase of the respiratory rate. This is generally observed immediately after the distortion of the respiratory curve, caused by the detonation, has disappeared, i. e. within 1 or 2 seconds after the detonation in most cases. The respiration rate is usually, even then, twice as great as before. Only in animals exposed to high maximum pressures and great impulses has a period of apnoea followed immediately after the detonation. The duration of the



apnoea has varied from a few seconds to more than one minute. It has often been hard to determine whether this has been an actual apnoea or only a respiration with exceedingly small amplitude. The slow, irregular respiration following the period of apnoea observed by KROHN *et coll.* does not appear regularly in this material where in several cases this period is abruptly interrupted by a highly accelerated respiration.

In animals which react with an instantaneous increase of the respiration rate the breathing is usually extremely irregular during the first 10 to 20 seconds both in regard to the frequency and amplitude and also in regard to the shape of each respiratory cycle. Later it is generally regular, rapid and shallow.

The amplitude of the respiration curve is usually considerably smaller after the detonation than before. A certain correlation exists between frequency and amplitude in such a way that the breathing becomes more shallow the more rapid it is. *In severely injured animals there is, however, a considerable reduction of the pulmonary ventilation.*

In the most severely injured animals the respiration ceases immediately, or almost immediately, after the detonation. Then it is possible to observe two or three very slow breaths with slight amplitude. *If the detonation is violent enough it is not unusual that the animal dies without having made any respiratory effort after the detonation,* an occurrence, which KROHN *et coll.* have stated as rare.

The respiratory changes are related in some way to the degree of lung lesion and thus they are dependent on the magnitude of the forces of pressure and impulse acting on the animal. *The most important factor seems to be the maximum pressure as the greatest changes in frequency and amplitude of the respiration have appeared in animals exposed to high maximum pressures in spite of the impulse having been comparatively small.* In very severely injured animals which, nevertheless, survive the period immediately after the detonation, respiration is slow and irregular and usually remains slow until death.

A period of apnoea immediately after the detonation exists mostly only in animals which have been exposed to high maximum pressures and great impulses, but seems not particularly common even then. It cannot be decided with certainty from the results of these experiments, why some animals have their respiration interrupted, while others, which have been exposed to a similar blast effect and

have obtained lung lesions of similar extent react by an instantaneous increase of the respiration rate. It will merely be pointed out here that most of the animals which have had their respiration interrupted have been struck by the blast wave with their respiration in expiratory position. In this connection it may be mentioned that HEAD (1889) found that a rapid inflation of the lungs immediately followed by deflation produces, if occurring during a phase of inspiration, a sudden shortening of this. If the inflation occurs during a phase of expiration the expiratory pause is usually quite considerably lengthened. He was also able to show that a momentary increase or decrease of the volume of the lung affects this pause, not only during the period of this change but even after the lungs had returned to their normal condition. The importance of the respiratory phase in this connection is not certain, however, and further investigations are required here as well as in regard to the importance of the respiratory phase for the extent of the lung lesion.

The pulmonary haemorrhages may cause a considerable decrease of the effective volume of the lung. Even the vessels of the lung being immensely dilated may noticeably intrude upon the pulmonary air space (DRINKER, PEABODY and BLUMGART 1922). The vital capacity is lowered, and the total ventilation of the lung reduced. In some of the most severely injured animals ventilation is quite negligible. This explains the apparently somewhat peculiar fact that in a number of cases where the animals had an exceedingly strained respiration using the respiratory muscles, including the auxiliary muscles, to their maximum capacity hardly any ventilation could be observed in the pneumogram. *Owing to the massive haemorrhages not only in the lungs but also in the upper respiratory passages the respiratory movements were followed only by a very slight ventilation.* These animals usually died within 5 minutes after the detonation. There were haemorrhages almost throughout the lungs, and the lungs did not collapse when the chest was opened (increased *viscous resistance* according to BAYLISS and ROBERTSON 1939; see also KROHN *et coll.* 1942).

The changes in respiration caused by the blast wave may be supposed in principle to be brought about in several different ways. The possibilities which should be discussed in this connection, are: a direct effect on the respiratory centre, and an effect caused by afferent impulses, mechanical as well as chemical, released in the lungs and the circulatory system.

A blast wave of sufficient force can no doubt be transmitted through the tissues of the body to the spinal canal and the central nervous system thus temporarily influencing the activity of vital centres.

YOUNG (1945) points out that the increase of pressure in thorax and abdomen caused by the detonation not only inhibits the venous reflux to the heart but also causes the blood stream to go in the opposite direction thus distending vena cava and the pulmonary veins. The increase of pressure in these veins propagates as far as to the central nervous system leading to an increase of the cerebrospinal fluid pressure (cf. also WHITEHORN *et coll.* 1947). By ligating or obliterating the jugular veins, on the other hand, this pressure increase could be almost entirely prevented (YOUNG).

This altered haemodynamic state may cause temporary changes in the activity of the respiratory- and circulatory centres. *But even if the shock wave directly affecting the central nervous system may play some part, the results, however, obtained by KROHN et coll. (1942) and the present author from experiments with vagotomized animals and on animals the bodies of which have been protected, except for the heads, from the blast wave, indicate that the respiratory changes chiefly are of reflex origin, the vagus nerve being the afferent path.*

The importance of vagus for regulating the respiration was shown through the well-known investigations carried out by HERING and BREUER (1868) and HEAD (1889), and our knowledge has been further enriched by a number of papers from different laboratories. Among all these investigations only a few will be mentioned here which seem to throw light on the changes in respiration caused by blast waves.

HAMMOUDA and WILSON (1932) have shown that an increased or decreased pressure on thorax caused an immediate increase or decrease respectively, of the respiratory rate. The changes appeared only if vagus was intact. They believe that the increase in frequency is due to the impulses from the stretch endings quickening the rhythm by cutting short the discharge and so hastening the recharging process. In experiments on rabbits ADRIAN (1933) was able to show that the compression of thorax produces an increased inspiratory effort owing to stimulation of deflation nerve-endings in the lungs. Similar results were obtained if the intra-pulmonary pressure was lowered by air being sucked from the respiratory passages. According to MOORE (1927) obstruction of one of the bronchi may cause tachypnoea which is suppressed if the vagus nerve on the same side is cut.

KROHN *et coll.* (1942) found that an increased afferent activity was maintained in vagus after the detonation, and that an immediate increase of the discharge in the diaphragm could be shown. The change in the respiration of animals injured by blast is supposed to be due to an increased stimulation of deflation endings, and to an increased sensitivity of stretch endings to distension of the lungs.

The haemorrhages no doubt play the greatest part in causing this effect, and the progressive increase of the respiration, usually seen during the first few hours after the detonation, seems to be a sign of the continued bleeding. The presence of a pronounced tachypnoea, even in animals with only insignificant lung haemorrhages, shows, however, that the direct distension of the lung (*volumen pulmonum auctum*, traumatic emphysema) is also of importance.

The vagal nerve endings in the lung are considered mainly localized to the walls of the alveolar ducts (cf. GORDH 1945) which are the most distensible parts of the lung. KROHN *et coll.*, however, basing their opinions upon investigations by PARTRIDGE (1939), suppose that the deflation endings may be proprioceptive fibres issuing from the blood vessels of the lung. In view of this the considerable distension of the capillaries as well as the smaller blood vessels in the blasted lung would possibly also be an important factor. It may also be pointed out here that BINGER, BROWN and BRANCH (1924) and PARTRIDGE (1935) were able to produce rapid, shallow respiration by obstructing the lung circulation by injecting a starch suspension into the blood vessels. HARRISON *et coll.* (1932) found that the reduction of the vital capacity caused by distension of the lung capillaries or by salt solution being injected into trachea, resulted in tachypnoea if vagus was intact.

Finally, it may be mentioned that MOORE and HARRISON (1939) have shown that atelectasis is an important cause of tachypnoea.

The exchange of gas in the lung is directly proportional to the partial pressure of the gas and to the size of the respiratory surface and reciprocally proportional to the thickness of the alveolar membrane, i. e. to the diffusion way (cf. KROGH 1915).

The respiratory surface is reduced by the haemorrhages and the diffusion way is lengthened. The gas exchange is extremely difficult in the severely injured lungs weighing two to three times more than normally, and this leads to a fairly pronounced anoxemia. The anoxemia has been verified by oximetry which has shown a reduc-

tion of the arterial oxygen saturation after the explosion. In chapter 16 below it will be proved, that the severely injured animals generally have a lowered alkali reserve. These changes may also cause an acceleration of the respiration.

Through extensive investigations carried out in a number of laboratories (HERING, HEYMANS, SCHMIDT, VON EULER, LILJESTRAND, ZOTTERMAN, and others) the importance of the chemoreflex regulation of the respiration brought about by the *carotid and aortic bodies*, has been explained (for references see, for instance, HEYMANS and BOUCKAERT 1939, GORDH 1945, BJURSTEDT 1946, and GERNANDT 1946).

The carotid bodies are extremely sensitive to oxygen lack, a fact shown by VON EULER, LILJESTRAND and ZOTTERMAN (1939). They found an increased discharge in the chemoreceptor fibres as soon as the arterial oxygen saturation was lowered below 96 per cent. In order to double the ventilation, however, the oxygen saturation must be less than 60 per cent (HARRISON *et coll.* 1932).

The slight increase of the respiration rate after the detonation seen also in some of the vagotomized animals may be explained as chemoreflex action caused by the hypoxia brought about by the lung haemorrhages, and the excess of carbon dioxide may stimulate the respiratory centre. BINGER, BREW and BRANCH (1924) have indeed shown that the tachypnoea which is caused by asphyxia of the respiratory centre can be abolished by bilateral vagotomy. HARRISON *et coll.* (1932), however, found that vagotomized animals were insensitive to changes of the vital capacity, but that they reacted by increased ventilation to chemical changes in the blood. These changes, however, could never cause the violent tachypnoea which could be brought about by a reduction of the vital capacity.

Experiments with animals bilaterally vagotomized and with denervated carotid sinuses carried out by the present author do not contradict the hypothesis that peripherally released chemoreflexes could be of importance here. Chemoreflex action caused by impaired gas exchange in the haemorrhagic lungs, however, cannot be the most important factor in causing accelerated respiration a fact seen in some animals which, in spite of only insignificant pulmonary haemorrhages, show a pronounced acceleration of the respiration due to distension of the lungs (for instance rabbit S 173, fig. 27 page 101).

With regard to the radical changes of the heart action and blood

pressure after the detonation which will be discussed in the following chapters, the correlation between blood pressure and respiration shown by HEYMANS and HEYMANS (1927) among many others, is also worth mentioning in this connection. HEYMANS was thus able to show (1928, 1929) that a lowering of the blood pressure produced a reflex hyperpnoea.

*When summarizing the results in this chapter it may be said that respiration is highly influenced by blast waves. The respiration rate is increased and the amplitude and total ventilation diminished. Shock waves with high max. pressures have the greatest effect, while those with low max. pressures but great impulses influence the respiration only more moderately. In animals exposed to high max. pressures as well as great impulses the respiration rate is usually only slightly increased owing to the extensive pulmonary haemorrhages. Immediately after the detonation respiration is usually quite irregular. In severely injured, surviving animals a period of respiratory stand-still lasting from a few seconds and up to more than one minute may be seen. It seems to occur preferably in animals struck by the shock wave with their respiration being in an expiratory position. It is not unusual for the most severely injured animals to die without having drawn one single breath after the detonation. In other cases there is a period of maximal respiratory strain, which, however, on account of the severe lung haemorrhages, does not result in any ventilation.*

*In animals bilaterally vagotomized before the detonation there is no increase or only an insignificant increase of the respiration rate. The amplitude, on the other hand, is increased.*

*Animals exposed to max. pressures and impulses high enough to cause immediate death show only little or no change of the respiration rate if their bodies, except for their heads, have been protected against the shock wave by a steel cylinder thus preventing pulmonary haemorrhages.*

## CHAPTER 13.

### The Effects of Blast Waves on Heart Action.

Earlier clinical observations and animal experiments have shown that macroscopical as well as microscopical lesions in the heart and

the larger blood vessels may be caused by the blast wave, but that such lesions are comparatively rare.

Earlier investigations into the effects of blast waves upon the normal physiological functions of the circulation are very few.

HOOKE (1924), and KROHN, WHITTERIDGE and ZUCKERMAN (1943) are apparently the only authors who have studied the heart action in blasting experiments.

HOOKE's investigations mainly concerned blood-pressure and no special attention was given to heart action, but he points out, however, that the heart rate increased in most of the animals after the detonation, a fact which was supposed to be due to a reduction of blood-pressure. The peripheral vagus mechanism was functioning, and stimulation of vagus produced instantaneous inhibition. The cardiac valves were intact and no pathological changes in the heart muscle could be seen.

The only more detailed study of the circulation in blasted animals has been carried out by KROHN *et coll.* (1942). They registered electrocardiograms with subcutaneous silver-foil electrodes in right shoulder region and left thigh. The electrocardiograph was placed 80 yards from the site of the explosion. Recordings could generally be obtained within one or two seconds after the detonation. The experiments carried out mainly to find out whether animals which were killed outright by blast had died from ventricular fibrillation gave, in the main, the following results:

In 15 animals exposed to blast pressures of the order of the  $P_{50}$  level (6 animals died) no changes of coordination could be found. Occasionally short periods of changes in the size of the QRS complex were found and sometimes a few ventricular extrasystoles were noticed. Only 2 of the animals showed a reduction of the heart rate of more than 7 per cent. The greatest reduction noticed was 20 per cent.

Animals exposed to such high maximum pressures as would cause almost instantaneous death (2—3 times the  $P_{50}$  level) showed a reduction of the heart rate by 60—70 per cent and also a reduction of the amplitude, but no alteration was observed in the coordination of the beat. All animals had obtained severe lung injuries.

The bradycardia after the detonation was found also in vagotomized or atropinized animals, but not, on the other hand, in animals whose thorax and abdomen had been protected by a steel cylinder.

Finally, it may be mentioned that CLARK and WARD (1943) in

their experiments on immersion blast injuries, referred to previously, found that the heart temporarily slowed up to half or less of its normal rate immediately after the animal had been struck by a shock wave with fatal effect.

The clinical observations which have been published concerning blast effects on the heart action are also very few. COHEN and BISKIND (1946) mention one case, a soldier who was 12 m away from an exploding V 1-bomb. He had regained consciousness 10 hours after the accident but his heart action and respiration were irregular. The systolic arterial blood-pressure was 150 mm Hg. and the diastolic pressure 75 mm Hg. The injured died on the fifth day.

BARROW and RHOADS (1944) and LEAVELL (1945) are apparently the only authors who have published electrocardiograms from human beings injured by blast. The main changes in the E. C. G. described by BARROW and RHOADS were low voltage and flattened QRS complexes. There was no arrhythmia or fibrillation. The pulse rate was less than 60 beats per minute in 26 per cent of the cases, less than 70 in 75 per cent, and less than 80 in 92 per cent of all the cases. In addition to this bradycardia a soft, easily compressible, collapsing peripheral pulse, was noticed. LEAVELL found an acute congestive heart failure in three cases of blast injury. In three other cases, also injured by blast, there were some abnormalities in the electrocardiogram, chiefly in the QRS complexes.

### Own investigations.

The examinations by HOOKER, and KROHN, WHITTERIDGE and ZUCKERMAN of the physiological changes of the circulation are in part incomplete but suggest, however, that far-reaching functional changes may result after exposure to blast waves. It was evident both from their experiments and from preliminary observations by the present author that a more or less pronounced bradycardia was the most outstanding functional change, and that in a violent explosion it could reach excessive degrees. It seemed plausible, therefore, that by studying the reactions of the circulatory system it would be possible to explain why some of the animals with only moderate lung lesions died, nevertheless, a few minutes after the detonation. Another interesting problem here is the reaction of the circulatory system to the sudden strong additional strain on the right ventricle when the lungs in a short time become more or less completely hepatized.

The heart action has been recorded with an electrocardiograph during and immediately after the detonation, according to the method described on page 32. In all these experiments the recording



has started 5 or 10 seconds before the detonation *and has continued without interruption till 30 to 60 seconds after the detonation, that is to say, during the time when the most apparent changes are seen.* After this, recordings have been taken at different times up to 24 hours after the explosion. The exposures immediately following the one made during and immediately after the detonation, have been taken at short intervals (varying between 15 seconds and 5 minutes). The changes in the electrocardiogram have all the time between the recordings been followed on the focussing glass of the cardiograph.

Before discussing the changes produced by the detonation the normal electrocardiogram of the rabbit will be given some attention.

### **The normal electrocardiogram of the rabbit.**

The electrocardiogram of the normal rabbit is, in many ways, similar to that of man. Some differences exist, however, the most apparent of which is the difference in heart rate which in the rabbit is considerably higher. According to SCHINZEL (1933) the heart rate of rabbits varies between 150 and 365 per minute. In the present material the heart rate in unanaesthetized animals varies between 210 and 300 with a mean value of  $252 \pm 6$  ( $\sigma = 27$ ) beats per minute, and in anaesthetized animals between 140 and 340 with a mean value of  $249 \pm 7$  ( $\sigma = 38$ ) beats per minute. The E. C. G. usually shows a certain left axis deviation, but in a number of cases there is instead a right axis deviation.<sup>1</sup>

More or less atypical E. C. G. can often be found in the rabbit without any detectable change in the heart. Thus a ventricular complex may be seen deviating in either direction from the normal, and also in the other waves (especially in the terminal complex) there may be smaller divergences from the normal (STRAUB 1909).

The R wave is generally not so large as in the human E. C. G., while the rabbit, on the other hand, often has a noticeably high T wave. The R or S wave may be notched without apparent pathological importance, a fact also pointed out by WIZER and HABAN (1939).

SCHINZEL, who has thoroughly studied the electrocardiograms of the small animals used in laboratory experiments, gives the following figures as regards

<sup>1</sup> In spite of some differences in the position of the rabbit heart as compared with the human heart it has seemed convenient to use the same terminology as in human electrocardiography. The terminology used is in accordance with that of BURCH and WINDSOR (1947).

the duration and size of the different waves. The auricular complex generally lasts 0.032—0.042 sec and the amplitude is 0.10—0.15 millivolts (if 1 cm corresponds to 1 millivolt). The distance between P and R (the P—R segment) is usually 0.021—0.032 seconds, and the P—R interval is 0.053—0.074 seconds. The R wave generally lasts 0.015—0.019 seconds and has an amplitude of 0.35—0.40 millivolts. Often a small negative S wave occurs which lasts about 0.006 seconds with an amplitude of 0.075—0.1 millivolts. No R—T segment (S—T segment) exists in the E. C. G. of the rabbit, as the R or S waves are immediately followed by the terminal wave T. According to SCHINZEL the T wave is always positive and lasts 0.095—0.11 sec with an amplitude of 0.22—0.27 millivolts.

According to LAMBERT (1937), the P wave may be small and diphasic or negative in lead I. In leads II and III, on the other hand, it is always positive. The QRS complex, as pointed out by SCHINZEL, merges into T without a distinct isoelectric distance, and consequently there is no S—T segment or only a very short one. The T wave is always positive in leads I and II, but may be either positive or negative in lead III.

#### **The electrocardiogram of blasted animals.**

Immediately after the detonation an often very pronounced distortion of the E. C. G. curves occurs probably partly due to potential fluctuations caused by movements of the electrodes at the moment of the detonation and partly to strong muscular movements and muscle tremor. That this latter factor is of great importance is shown by the fact that the disturbances were considerably smaller in one case where the electrodes had been inserted in a dead rabbit during the detonation. In unanaesthetized animals very great disturbances often occur during the first 30 to 60 seconds after the detonation owing to action potentials from the extremity muscles. These disturbances are in many cases so pronounced that the curve has been useless for a detailed analysis. In cardiograms taken later than one minute after the detonation they have often quite disappeared. They do not exist at all in anaesthetized animals.

In the following an account will be given of some effects upon the heart action of blast waves with different pressures and impulses. *The material has been divided into four groups in the same way as in the respiration chapter* (see above, page 98). The same limit value (4 kgf/cm<sup>2</sup>) for the maximum pressure has been used, but that for the impulse has been increased to 1.5 gf.sec/cm<sup>2</sup>. No detailed accounts

will be given here of all the different changes in the coordination and the size and shape of the different waves and complexes, which often occur especially in the severely injured animals exposed to blast waves with high maximum pressures. It may also be pointed out here that the electrocardiograms shown in the figures below are, with a few exceptions, intended to give only a general survey of the changes — mainly of the heart rate — but not the details of the different waves. The author hopes, however, to be able to give a special analysis of these changes in following papers.

### 1. The effects of low maximum pressures and small impulses.

Already from a few preliminary experiments it was clear that the heart action was practically unaffected by a blast wave with low pressure and small impulse. Because of this only a few experiments have been carried out within this group.

The only noticeable change in these animals was a rather insignificant bradycardia during the seconds immediately after the detonation succeeded by a likewise rather insignificant rise in frequency over the initial value. An example of this is animal S 130 which had been exposed in the field (group type A) to a detonation of 250 g of *nitrolite* at a distance from the charge of 1.36 m. The maximum pressure was  $2.68 \pm 0.4$  kgf/cm<sup>2</sup> and the impulse 0.125 gf · sec/cm<sup>2</sup>. The heart rate was 260 beats per minute before the detonation but rose rapidly to 270 beats per minute after the detonation. One minute and a half after the detonation it was 260, and two minutes and a half after it, it was 250 beats per minute.

In three out of four animals exposed to blast in the field (group type B) the heart rate 20 minutes after the detonation was somewhat lower than the initial value. In two of the animals examined 18 hours after the explosion the heart rate was still somewhat reduced (see table 22). All these animals were anaesthetized.

No changes could be noticed in the coordination or in the size or shape of the E. C. G. waves.

### 2. The effects of high maximum pressures and small impulses.

This group comprises 25 animals exposed to blast in the field (group type A). They have all been unanaesthetized. Five of the animals died within a few minutes after the detonation.

TABLE 22. Data concerning heart rate, lung injury etc. of four rabbits exposed in the field to shock waves with low max. pressure and small impulse.

Rabbit	Weight of charge kg	Distance from charge m	Max. pressure kgf/cm <sup>2</sup>	Impulse gf·sec/cm <sup>2</sup>	Heart rate			Quotients of lung injury			
					Before detonation	20 min after detonation	18 hours after detonation	Haemorrhage (+ oedema)		Increase of lung volume (emphysema)	
								Left lung	Right lung	Left lung	Right lung
G 22	7.25	3.54	3.2	0.63	360	290	300	0.86	0.85	1.06	1.17
G 24	7.25	6.16	1.8	0.43	310	280	300	0.95	1.14	1.08	1.09
G 28	100	16.23	1.0	0.95	260	260	—	0.95	1.39	1.17	1.16
G 38	29.25	12.03	1.8	0.6	270	250	—	0.99	0.97	1.01	0.97

TABLE 23. Mean value of heart rate during the first few minutes after the detonation in rabbits exposed to shock waves with high max. pressure ( $>4$  kgf/cm<sup>2</sup>) and small impulse ( $<1.5$  gf·sec/cm<sup>2</sup>).

	Before detona- tion	Time after detonation in sec										1 $\frac{1}{2}$ min	2 $\frac{1}{2}$ min	3 $\frac{1}{2}$ min	5 $\frac{1}{2}$ min
		0—3	3—6	6—9	9—12	12—15	15—18	18—21	21—24	24—27	27—30				
$n$	18 247	9 144	14 109	16 79	17 87	16 99	17 122	18 122	17 122	16 135	10 155	15 181	10 187	10 195	5 172
$\epsilon(M)$	5.8	20.5	12.9	13.2	16.2	18.2	15.8	14.1	15.0	16.2	16.7	21.6	22.3	27.8	31.5
$\sigma$	24.5	61.0	48.4	52.6	66.6	72.6	65.0	60.0	61.8	64.9	55.5	83.7	74.4	92.6	70.5

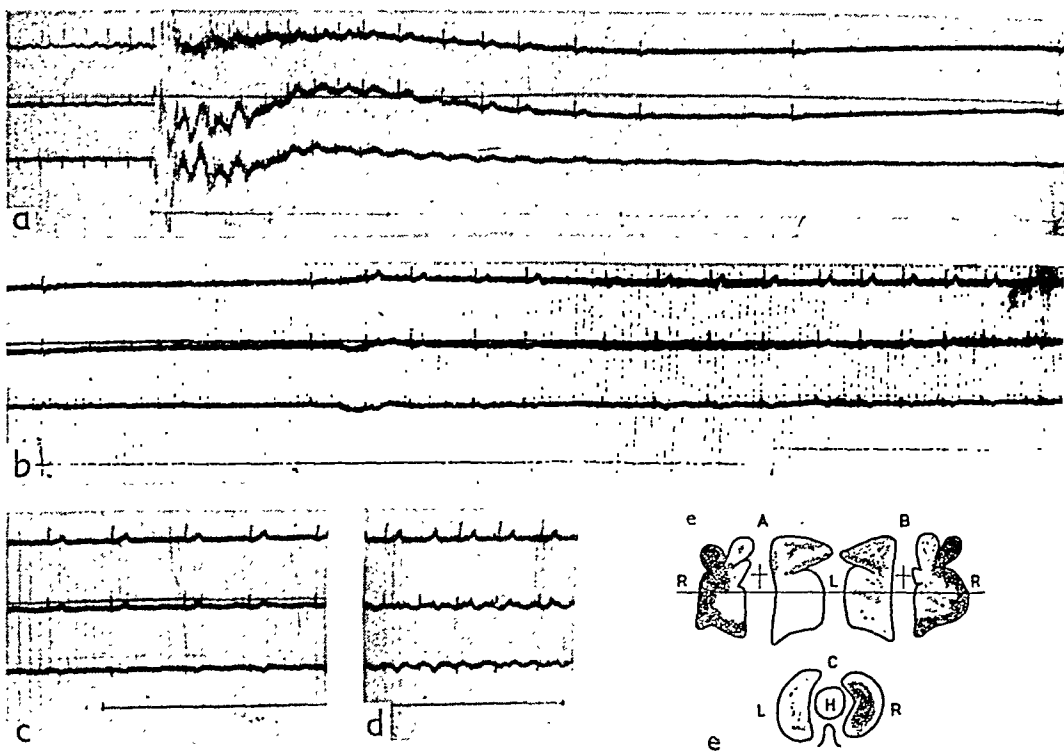


Fig. 31. Electrocardiogram of rabbit S 119, exposed to a max. blast pressure of 11.9 kgf/cm<sup>2</sup> and an impulse of 0.97 gf · sec/cm<sup>2</sup>. *a.* Before and until 12 sec after the detonation (detonation at arrow). *b.* 12—25 sec, *c.* 1.5 and *d.* 3.5 min after the detonation. Time marking 0.1 sec. *e.* Lungs with haemorrhages.

The most noticeable change in the electrocardiogram is the almost instantaneous bradycardia which may be quite prominent (see figs 31 and 32). This bradycardia, as shown in fig. 33 and table 23, reaches its maximum already 9 seconds after the detonation. In several of the animals the rate has then dropped from 250 or 300 beats per minute to 20 or 40 per minute. The rate then increases again rapidly to begin with, but it will be some time before the initial value is reached. Still 5 minutes or more after the detonation the average heart rate is 185 beats per minute as against 245 before the detonation. The dispersion, however, is quite considerable, and in some cases only a fairly slight reduction is seen.

In single animals the slowing-up of the heart rate sets in somewhat later and is most pronounced 20 or 30 seconds after the detonation.

In animals which die within some minutes after the detonation the rate increases again slightly after the initial bradycardia, but does not reach the initial value before the final slowing-up of the heart action begins.

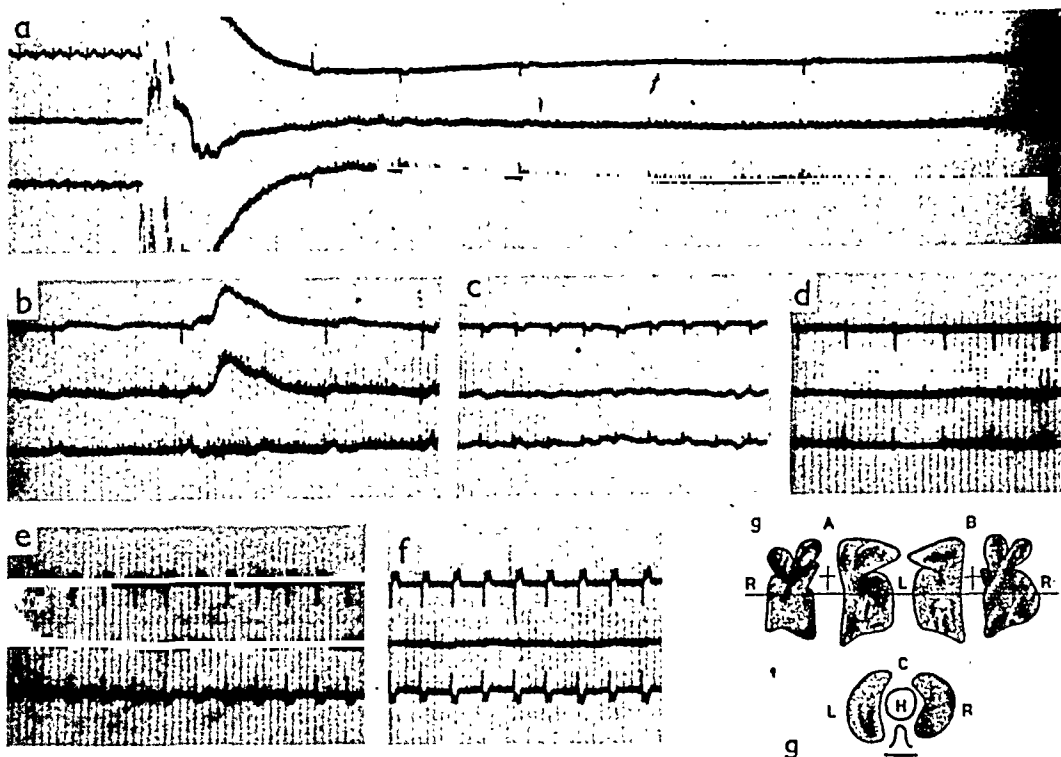


Fig. 32. Electrocardiogram of rabbit S 137, exposed to a max. blast pressure of 13.3 kgf/cm<sup>2</sup> and an impulse of 1.13 gf · sec/cm<sup>2</sup>. a. Before and until 11 sec after the detonation (detonation at severe distortion). b. 27—32 sec, c. 2, d. 4, e. 5 and f. 6 min after the detonation. Time marking 0.1 sec. g. Lungs with haemorrhages.

As an example of changes which may occur, a tabular description is given below of the changes in the electrocardiograms of three of the animals belonging to this group. In all cases the distance from charge was 1.36 m and the weight of charge 0.550 kg.

#### *Rabbit S 118.*

Maximum pressure:  $12.5 \pm 2$  kgf/cm<sup>2</sup>

Impulse: 1.12 gf · sec/cm<sup>2</sup>

Lung changes: Quotients of haemorrhage. Left: 1.40. Right: 2.56.

Quotients of volume increase: Left: 1.67. Right: 1.52.

#### *The electrocardiogram:*

Before the detonation: Heart rate: 300/min. Right axis deviation.

After the detonation:

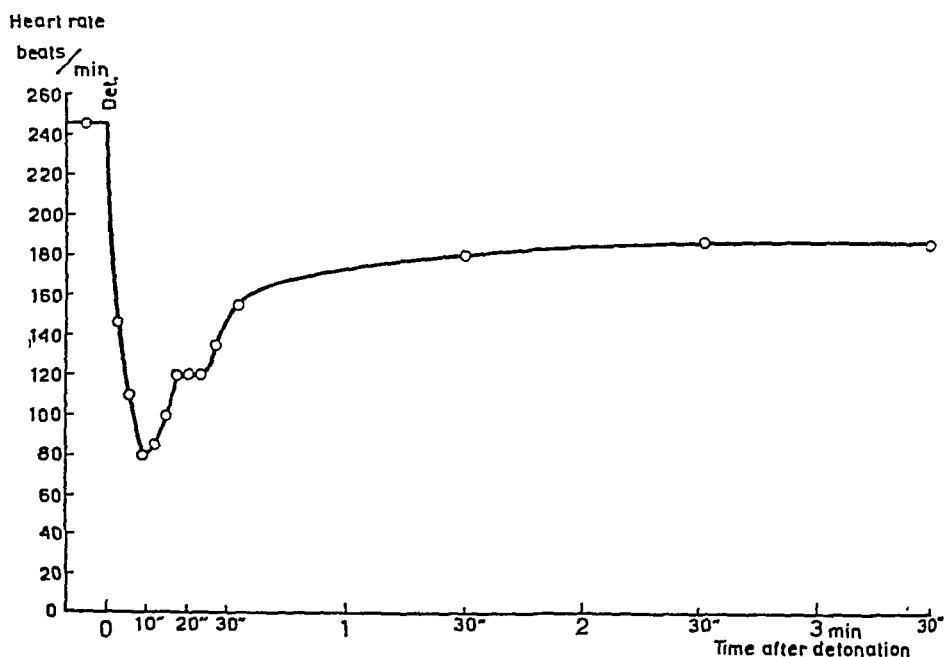


Fig. 33. Mean value of heart rate of animals exposed to high max. pressure and small impulse.

Time interval	Heart rate	Changes in the E. C. G.
0—3 sec	Cannot be determined	Cannot be determined
3—6	180	Unequal beats.
6—9	approx. 60	Completely irregular rythm with unequal beats.
9—12	Only one or two slight contractions can be noticed, and the condition must be regarded as an almost complete ventricular standstill.	
12—15	Only one definite ventricular contraction until 14.8 seconds, when a double extrasystole occurs.	
15—18	Extremely weak impulses can be noticed, but there is only one ventricular complex.	
18—21	Two ventricular complexes and one extrasystole corresponding to a frequency of 60/min.	
21—24	Frequency of definite ventricular contractions: 40/min.	
24—27	» » » » » : 40/min.	

In the following exposures the curves are technically useless. The animal survived the detonation.

*Rabbit S 119* (see fig. 31).

Maximum pressure:  $11.9 \pm 1.9$  kgf/cm<sup>2</sup>.

Impulse: 0.97 gf · sec/cm<sup>2</sup>.

Lung changes: Quotients of haemorrhage: Left: 1.02. Right: 1.73.  
 Quotients of volume increase: Left: 1.29. Right: 1.19.

*The electrocardiogram:*

Before the detonation: Heart rate: 210/min. Left axis deviation, depending on the position of the heart.

After the detonation:

Time interval	Heart rate	Changes in the E. C. G.
0—3 sec	180	Sinus arrhythmia. S <sub>III</sub> has changed from neg. (—4 mm), via diphasic state, to low pos.
3—6	100	Sinus arrhythmia. Interval between the complexes tending to become longer and longer. T <sub>I</sub> pos.
6—9		The bradycardia reaches its maximum. Intervals between the heart beats 3.7 and 3.2 sec respectively.
9—12	40	
12—15		T <sub>I</sub> is now definitely neg.
15—18	100	Moderate sinus arrhythmia.
18—21	90—100	Insignificant sinus arrhythmia.
21—24	100	Slight sinus arrhythmia. T <sub>I</sub> showing tendency to increase and to become accentuated.
24—27	110	
27—30	130	T <sub>I</sub> now + 3 mm.
1½ min	80	
3½	120	Slight sinus arrhythmia exists. T <sub>I</sub> becomes more and more accentuated and higher (+4 mm).

The animal was killed three hours after the detonation.

*Rabbit S 120.*

Maximum pressure:  $10.9 \pm 1.7$  kgf/cm<sup>2</sup>

Impulse: 0.94 gf · sec/cm<sup>2</sup>

Lung changes: Quotients of haemorrhage: Left: 1.05. Right: 1.89.  
 Quotients of volume increase: Left: 1.28. Right: 1.37.

*The electrocardiogram:*

Before the detonation: Heart rate: 250/min. Left axis deviation, highly probably depending on the position of the heart. P<sub>III</sub> and T<sub>III</sub> neg.



After the detonation:

Time interval	Heart rate	Changes in the E. C. G.
0—3 sec	80	T <sub>III</sub> has changed from low neg. (before the detonation) to fairly high pos.
3—6	60	T <sub>III</sub> is now definitely neg.
6—9	40	Still exceedingly irregular rhythm with pronounced bradycardia. Here is an interval of no less than 3.2 sec between 7.2 and 10.4 sec.
9—12	40	
12—15	70	Still sinus arrhythmia. T <sub>III</sub> remaining neg.
15—18	100	
18—21	110	
21—24	110	
24—27	90	Still considerable sinus arrhythmia.
27—30	120	
1½ min	100	An extrasystole occurs. T <sub>III</sub> distinctly accentuated, neg.
2½	220	T <sub>III</sub> becoming less accentuatedly neg.
3½	260	An extrasystole of ventricular type with compensatory pause occurs.
4½		Regular bigeminy with extrasystoles of similar origin as the above mentioned.

The animal was killed approximately 15 min after the detonation.

The rhythm is often irregular during the first 30 seconds after the detonation. There is generally a sinus arrhythmia which, in a number of cases, is transformed into completely irregularized rhythm with unequal beats.

Extrasystoles, mostly of ventricular type, exist in the E. C. G. of at least 12 of the 20 animals of this group. In one of the animals (rabbit S 120) they turned into regular bigeminy approximately 4½ min. after the detonation.

It is also noticeable how quickly the different waves change in size and shape and from positive to negative, or vice versa.

Particularly severe changes are seen in the *electrocardiogram* of rabbit G 32 exposed to a charge of 0.250 kg at a distance of 1.02 m and to a maximum pressure of 20.9 kgf/cm<sup>2</sup> and an impulse of 1.36 gf·sec/cm<sup>2</sup>. The *electrocardiogram* (fig. 34) shows left axis deviation before the detonation, regular rhythm and a heart rate of 240/min.

Immediately after the detonation very severe changes occur. Here the monophasic curves are seen which usually signify the dying heart. In this case the monophasic curve is almost entirely of the right-side

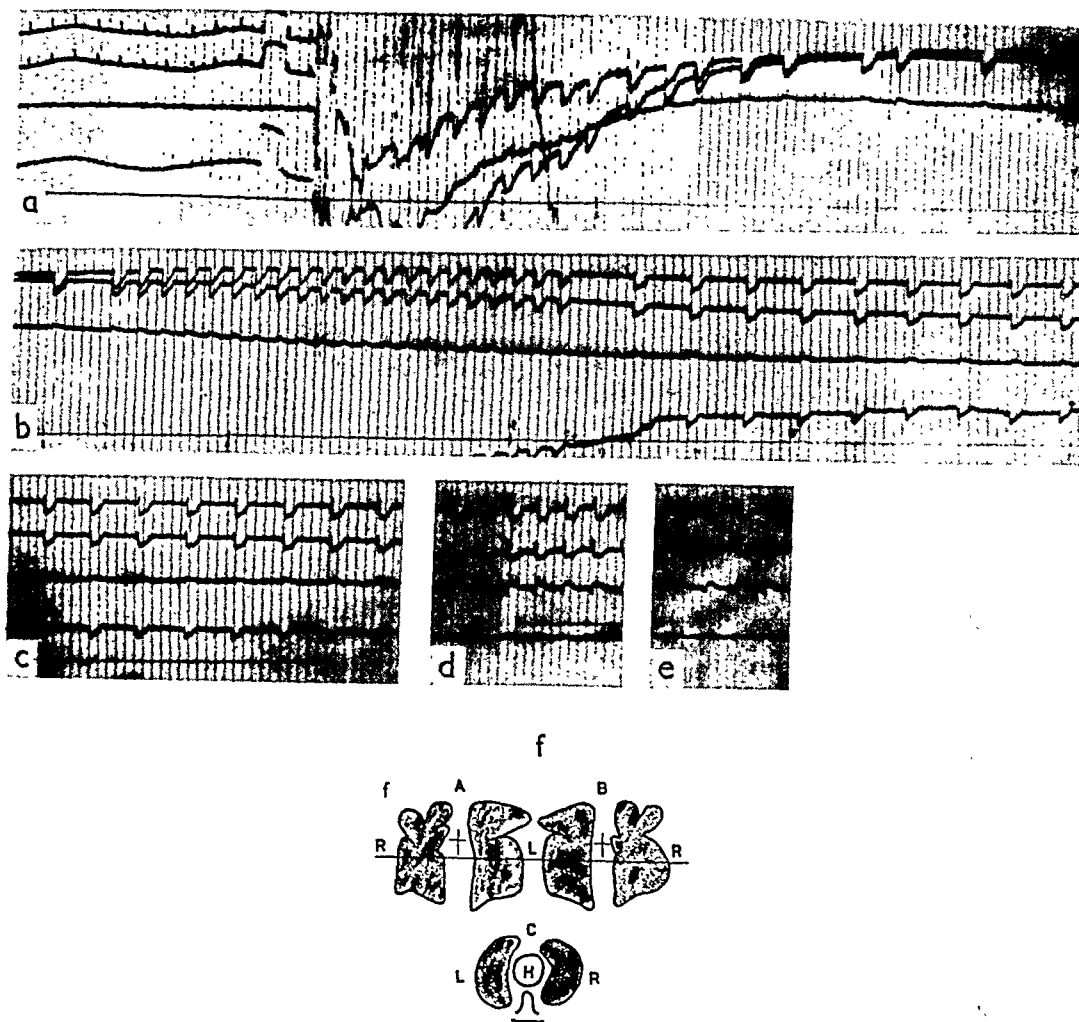


Fig. 34. Electrocardiogram and respiration of rabbit G 32, exposed to a max. blast pressure of  $20.9 \text{ kgf/cm}^2$  and an impulse of  $1.36 \text{ gf} \cdot \text{sec/cm}^2$ . a. Before and until 8 sec after the detonation. Calibration immediately before the distortion caused by detonation. b. 7—18 sec, c. 18—21 sec, d. 1.5 and e. 3.5 min after the detonation. Time marking 0.1 sec. f. Lungs with haemorrhages.

type, and there are no P waves. This state continues during increasing bradycardia for the next 6 seconds. P waves then begin to appear partly coordinated with QRS complexes which still show the severe changes mentioned above. The frequency is now 120/min. 7.7 seconds after the detonation a *ventricular flutter* begins which, however, lasts only 4.6 sec and has a frequency of 260/min. 12.6 seconds after the detonation the ventricular flutter, after a pause of 0.6 sec, changes into an almost regular normal rhythm. The auriculoventricular conduction is normal. There are still severe changes, however,

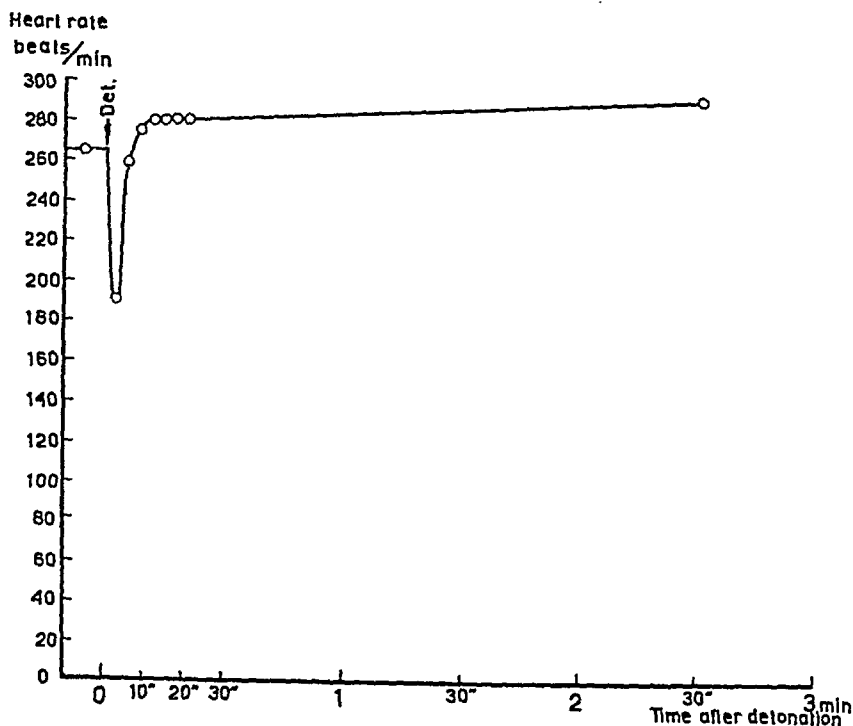


Fig. 35. Mean value of heart rate of animals exposed to low max. pressure and great impulse.

now of *right bundle branch block* type, which continue without variation for the next 10 seconds. The heart has thus been almost entirely at its end but recovers slightly and gradually while showing very severe lesions.

One minute and a half after the detonation changes are seen which show even clearer that the above-mentioned right bundle branch block has probably been caused by considerable lesions in the posterior wall of the heart. Two minutes later it is still the same. The frequency is 180/min. *Respiration ceased immediately after the detonation, and the heart action after approximately 10 min.*

### 3. The effects of low maximum pressures and great impulses.

This group consists of 11 animals all of which have been anaesthetized before being exposed in the detonation chamber. All these animals survived the detonation.

The E. C. G. changes are quite insignificant in this group, compared to those of the previous group. An almost momentary slowing-up of the heart rate occurs also in these animals, but is much less pro-

TABLE 24. Mean value of heart rate during the first few minutes after the detonation, of rabbits exposed to shock waves with low max. pressure ( $<4$  kgf/cm<sup>2</sup>) and great impulse ( $>1.5$  gf·sec/cm<sup>2</sup>).

	Before detonation	Time after detonation in sec							2 $\frac{1}{2}$ min
		0—3	3—6	6—9	9—12	12—15	15—18	18—21	
<i>n</i>	11	10	11	11	11	10	8	8	6
<i>M</i>	266	191	259	274	282	280	278	280	290
$\varepsilon(M)$	18.5	27.4	17.4	15.1	15.1	16.3	19.9	19.7	23.3
$\sigma$	61.4	86.7	57.8	50.2	50.0	51.6	56.2	55.6	55.8

nounced here, as shown in fig. 35 and table 24. The heart rate drops within the three first seconds from an average of 245/min to 190/min. In one of the animals (rabbit K 3) no ventricular complexes can be seen during these three first seconds, except towards the end when there are some slight muscle contractions.

The bradycardia is of very short duration and in some cases the heart rate is the same as or even somewhat greater than before the detonation. Already 6 seconds after the detonation the rate has again increased to an average of 200/min, and after a further 3 seconds it has exceeded the initial value, the average being 275/min.

In three animals exposed in the field (group type B) to a blast wave with low maximum pressure but relatively great impulse, the rate during several hours after the detonation was somewhat lower than before (see table 25).

Occasional extrasystoles of ventricular type exist in the cardiograms from 3 of the 11 animals within this group (animals K 11, K 14 and K 15). Hardly any other changes are seen.

#### 4. The effects of high maximum pressures and great impulses.

Twenty-three animals in all, anaesthetized with urethane, have been exposed in the detonation chamber to blast waves with high maximum pressures and great impulses. Seven of the animals died within 30 minutes. One animal died approximately 2 $\frac{1}{2}$  hours after the detonation.

Also in this group a momentary reduction of the heart rate is the most prominent change in the electrocardiograms. Three sec after the detonation the heart rate has dropped from an average of 255/

TABLE 25. Data concerning heart rate, lung injury etc. of three rabbits exposed in the field to shock waves with low max. pressure ( $<4$  kgf/cm<sup>2</sup>) and great impulse ( $>1.5$  gf·sec/cm<sup>2</sup>).

Rabbit	Weight of charge kg	Distance from charge m	Max. pressure kgf/cm <sup>2</sup>	Impulse gf · sec/cm <sup>2</sup>	Heart rate			Quotients of lung injury			
					Before detonation	20 min after detonation	4 hours after detonation	Haemorrhage (+ oedema)		Increase of lung volume (emphysema)	
								Left lung	Right lung	Left lung	Right lung
G 25	100	14.42	1.75	1.78	240	240	200	1.03	1.18	1.05	0.92
G 26	100	13.28	2.5	2.65	280	240	—	1.19	1.26	1.21	1.14
G 27	100	12.48	3.7	3.6	210	180	180	2.12	1.37	1.52	1.47

TABLE 26. Mean value of heart rate during the first few minutes after the detonation, of rabbits exposed in the detonation chamber to shock waves with high max. pressure ( $>4$  kgf/cm<sup>2</sup>) and great impulse ( $>1.5$  gf·sec/cm<sup>2</sup>).

	Before detona- tion	Time after detonation in sec										$1\frac{1}{2}$ min	$2\frac{1}{2}$ min	$3\frac{1}{2}$ min	$5\frac{1}{2}$ min	$6\frac{1}{2}$ min
		0—3	3—6	6—9	9—12	12—15	15—18	18—21	21—24	24—27	27—30					
$n$	16	16	16	16	15	14	12	12	10	6	4	8	8	11	7	4
$M$	255	155	198	206	219	218	219	214	197	167	153	216	204	186	170	240
$\varepsilon(M)$	9.9	15.4	11.7	11.7	12.1	9.5	10.1	12.5	11.3	25.2	32.0	12.2	22.3	22.5	21.9	10.8
$\sigma$	39.6	61.4	46.8	46.8	46.1	35.6	35.0	43.4	35.6	61.9	64.0	34.6	63.2	74.6	57.0	21.6

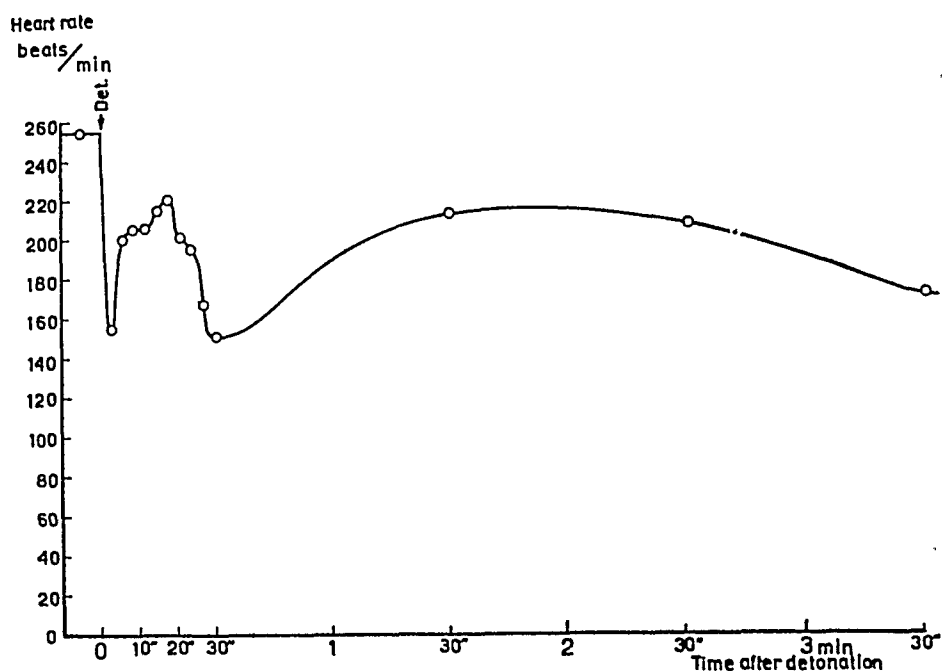


Fig. 36. Mean value of heart rate of animals exposed to high max. pressure and great impulse.

min to 155/min (fig. 36; table 26). Then it rises again fairly rapidly but does not generally reach the initial value until some time after the detonation. In one animal (K 20) no QRS complexes occurred until about 3 seconds after the detonation.

In five other animals exposed in the field (group type B) to a blast wave with high pressure and great impulse the rate two to six hours after the detonation was considerably lower than before the detonation. In two of the animals it was again normal after 18 and 24 hours (see table 27).

Extrasystoles of ventricular type occur with certainty in at least 7 of the 23 animals. In some other cases it is difficult to decide, owing to technical disturbances, whether extrasystoles exist or not. They may appear at any time after the detonation, immediately after it (e.g. K 31 which has an extrasystole 1.5 seconds after the detonation) or several minutes after it (e.g. K 40 which has an extra beat 22 seconds after the explosion and one after approximately 3 minutes). In rabbit K 32 (fig. 37) three extra beats occur between 5.7 and 7.4 seconds after the detonation.

In several cases E. C. G. changes are found suggesting lesions in the myocardium of infarct type.

TABLE 27. Data concerning heart rate, lung injury etc. in five rabbits exposed in the field to shock waves with high max. pressure ( $>4$  kgf/cm<sup>2</sup>) and great impulse ( $>1.5$  gf·sec/cm<sup>2</sup>).

Rabbit	Weight of charge kg	Distance from charge in	Max. pressure kgf/cm <sup>2</sup>	Impulso gf · sec/cm <sup>2</sup>	Heart rate				Quotients of lung injury				
					Before detonation	Time after detonation			Haemorrhage (+ oedema)	Increase of lung volume (emphysema)			
						20 min	2—6 hours	18 hours		24 hours	Left lung		Right lung
G 15	20.25	7.23	6.3	1.76	320	190	200	—	320	1.74	2.76	0.95	0.93
G 21	7.25	3.54	11.5	1.52	240	200	—	240	—	0.94	1.63	1.01	0.90
G 39	20.25	6.66	8.4	3.4	310	230	200	—	—	2.68	2.81	1.57	1.62
G 40	20.25	7.68	5.3	2.28	300	240	240	—	—	1.20	1.66	1.34	1.17
G 41	20.25	7.68	5.3	2.28	240	240	230	—	—	1.22	2.20	1.21	1.29

TABLE 28. Mean value of heart rate during the first few minutes after the detonation, of rabbits which after *bilateral vagotomy* have been exposed in the detonation chamber to shock waves with high max. pressure ( $>4$  kgf/cm<sup>2</sup>) and great impulse ( $>1.5$  gf·sec/cm<sup>2</sup>).

		Before detonation	Time after detonation in sec										
			0—3	3—6	6—9	9—12	12—15	15—18	18—21	21—24	24—27	27—30	1 $\frac{1}{2}$ min
$n$	14	8	14	14	13	14	14	13	11	9	6	11	
$M$	296	284	288	274	270	274	274	280	285	291	273	247	
$\varepsilon(M)$	10.5	21.0	12.6	13.2	14.1	11.2	11.1	12.3	13.8	16.3	17.4	25.7	
$\sigma$	39.3	59.5	47.1	49.5	51.0	41.8	41.5	44.3	45.9	48.9	42.7	85.6	

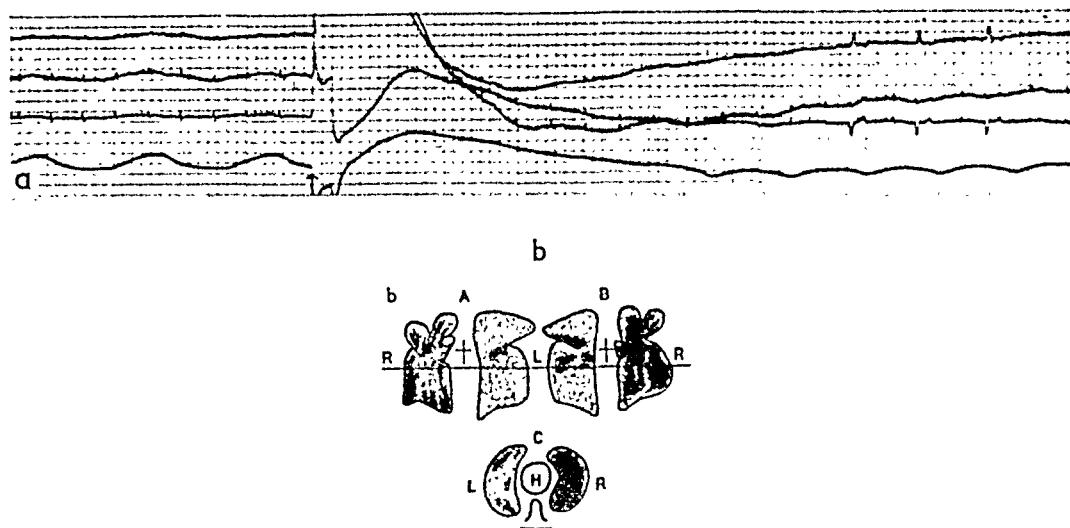


Fig. 37. Electrocadiogram and respiration of rabbit K 32, exposed to a max. blast pressure of  $11.9 \text{ kgf/cm}^2$  and an impulse of  $20.5 \text{ gf} \cdot \text{sec/cm}^2$ . *a.* Before and until 8 sec after the detonation (detonation at arrow). Between 5.7 and 7.4 sec after the detonation three extra systola are seen. Time marking 0.1 sec. *b.* Lung haemorrhages.

In the animals which die some minutes after exposure there are generally no particularly noticeable changes at first, except the usual bradycardia. But when the heart action begins to fail a more and more increasing block occurs with an ever increasing interval between the ventricular complexes and mostly well-preserved P waves. Towards the end the ventricular complexes usually consist of monophasic deflections which often are high and accentuated (e.g. K 28, fig. 38).

*In animals protected except for their heads by a steel cylinder and exposed in the detonation chamber to high max. pressures and great impulses there is no bradycardia, except during the first two or three sec. Immediately after this period, during which there are some disturbances in the electrocardiograms, the heart rate is the same or even greater than before the detonation. During the period of observation the rate remained higher than before the detonation. This acceleration may probably be due to liberation of adrenaline.*

### The effects on heart action in vagotomized animals.

As a link in the investigations of the effects of blast waves upon the heart and circulation a study has been made of the changes in the electrocardiograms of a number of animals on which bilateral vagotomy has been performed before the detonation.



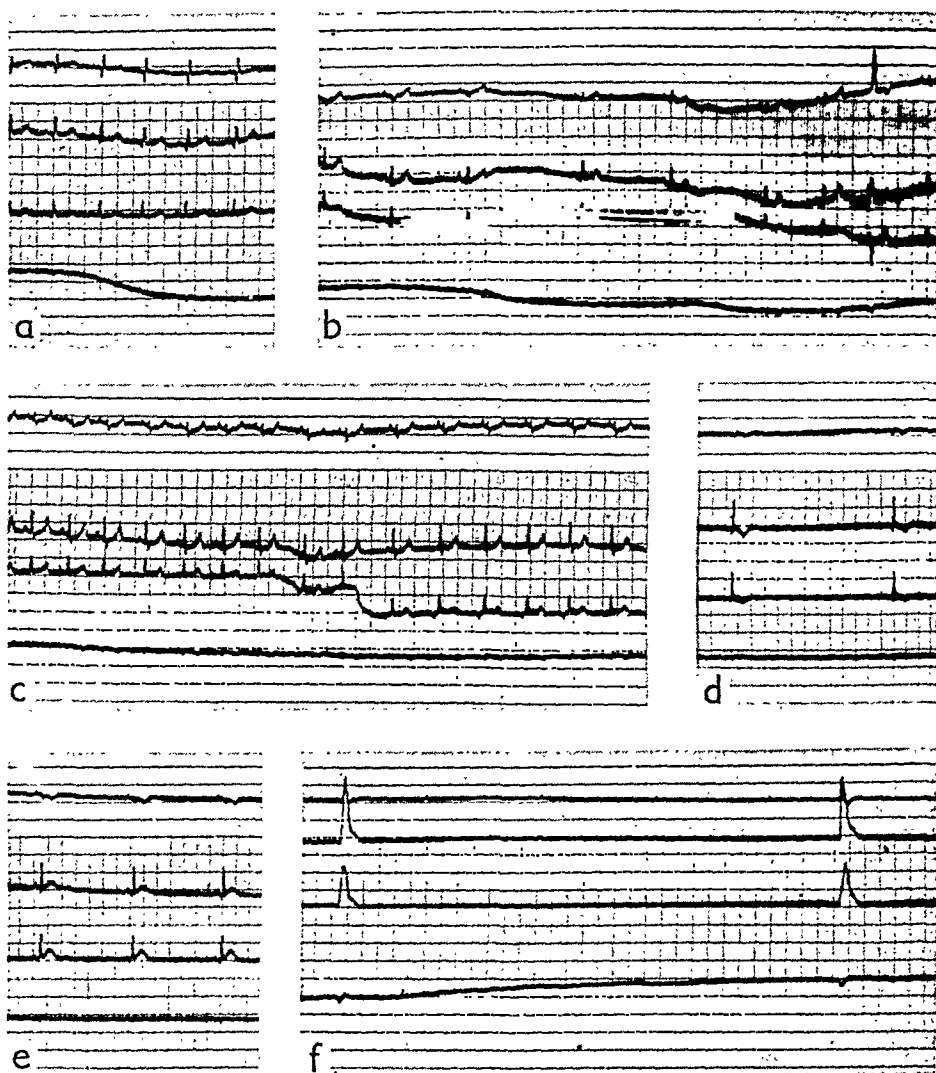


Fig. 38. Electrocardiogram (the three upper curves) of rabbit K 28, exposed to a max. blast pressure of  $14.7 \text{ kgf/cm}^2$  and an impulse of  $27.8 \text{ gf} \cdot \text{sec/cm}^2$ . *a.* Immediately before detonation. *b.* 5—9 sec, *c.* 23—27 sec, *d.* 1.5, *e* 2.5 and *f.* 3.5 min after the detonation. Time marking 0.1 sec.

These experiments comprise 15 animals. All blastings have been carried out in the detonation chamber. Fourteen animals have been exposed to a blast wave with high maximum pressure and great impulse. In one case, owing to incomplete detonation a maximum pressure of only  $3.8 \text{ kgf/cm}^2$  and an impulse of  $3.2 \text{ gf} \cdot \text{sec/cm}^2$  was obtained. No changes whatsoever appeared in the electrocardiogram of this animal.

The pronounced bradycardia appearing momentarily in non-vago-

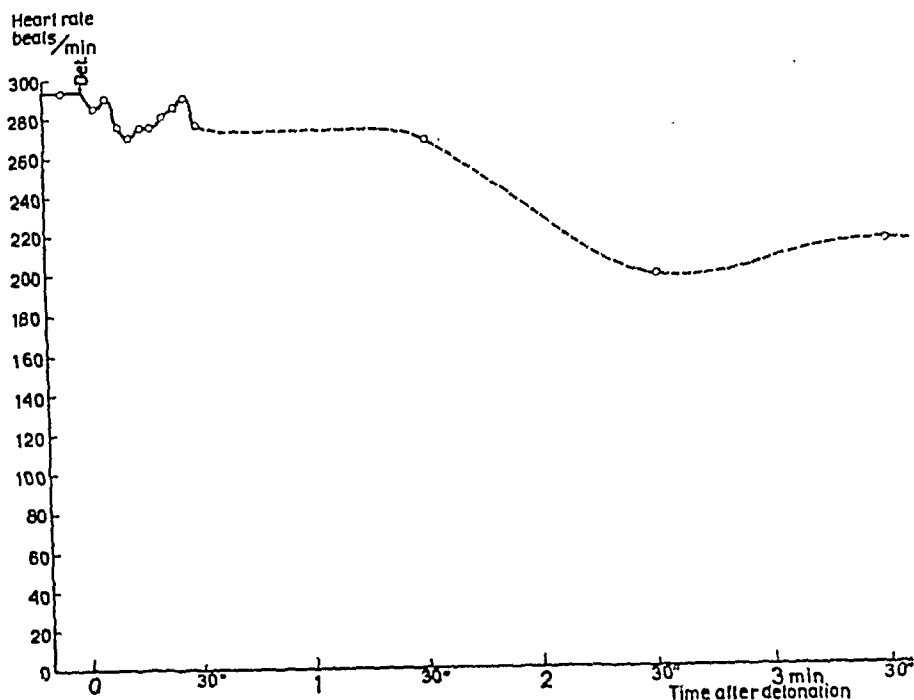


Fig. 39. Mean value of heart rate of animals exposed after *bilateral vagotomy* to high max. blast pressure and great impulse.

tomized animals after the detonation does not appear at all, or only to a very small extent, in vagotomized animals. This is shown in table 28 and fig. 39, which give the mean value of the heart rate in the 14 animals exposed to high pressures and great impulses.

Only in one case (animal K 41, see below) the heart rate has been reduced by more than 20 per cent within 30 seconds after the detonation. In three cases the rate 5 seconds after the explosion is even greater than immediately before.

The heart rate is lowest between 9 and 12 seconds after the detonation. The difference between the average obtained then (270/min) and the average frequency before the detonation (295/min) is statistically insignificant ( $0.2 > P > 0.1$ ).

The slowing up of the heart rate, which begins 1.5 minutes after the detonation and is marked by the dotted part of the curve (fig. 39), is due to the fact that some of the animals died a short time after the detonation with increasing bradycardia. All these animals have been included in this diagram.

Several of the animals show changes in the electrocardiograms which suggest severe lesion in the myocardium and the conduction

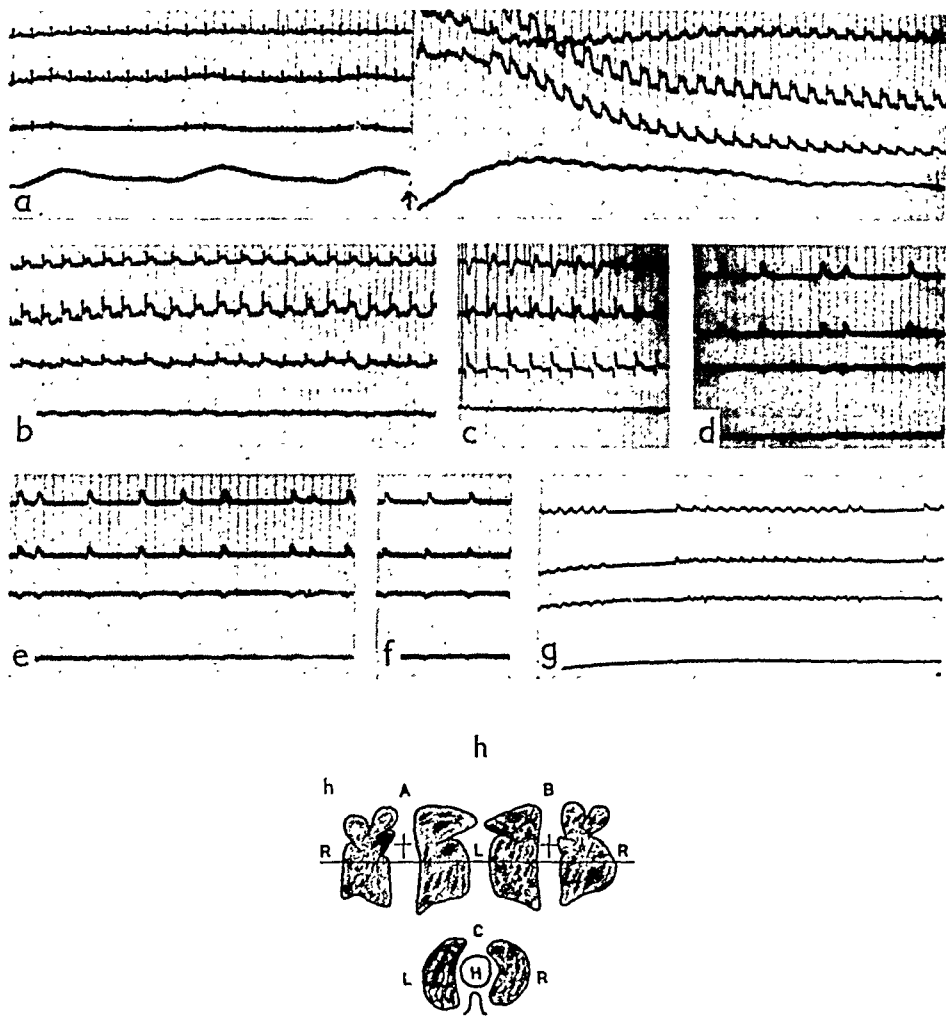


Fig. 40. Electrocadiogram and respiration of rabbit K 17, exposed after *bilateral vagotomy* to a max. blast pressure of 13.9 kgf/cm<sup>2</sup> and an impulse of 14.7 gf·sec/cm<sup>2</sup>. *a.* Before and until 6 sec after the detonation (detonation at arrow). *b.* 12—17 sec, *c.* 1.5, *d.* 2.5, *e.* 3.5, *f.* 10 and *g.* 11 min after the detonation. Time marking 0.1 sec. *h.* Lungs with haemorrhages.

system. In animal K 46, a high take-off to the T wave exists in leads II and III. This is highest approximately 20 seconds after the detonation. When the animal dies after about 3 minutes a few great positive, accentuated and notched deflections appear in pairs 3.5 minutes after the explosion (*bigeminy*). In animal K 50 there is a regular *bigeminy* 11 minutes after the explosion. Only the second beat in every pair has a P wave.

Rabbit K 17, which has been exposed to a maximum pressure of 13.9 kgf/cm<sup>2</sup> and an impulse of 14.7 gf·sec/cm<sup>2</sup>, shows considerable

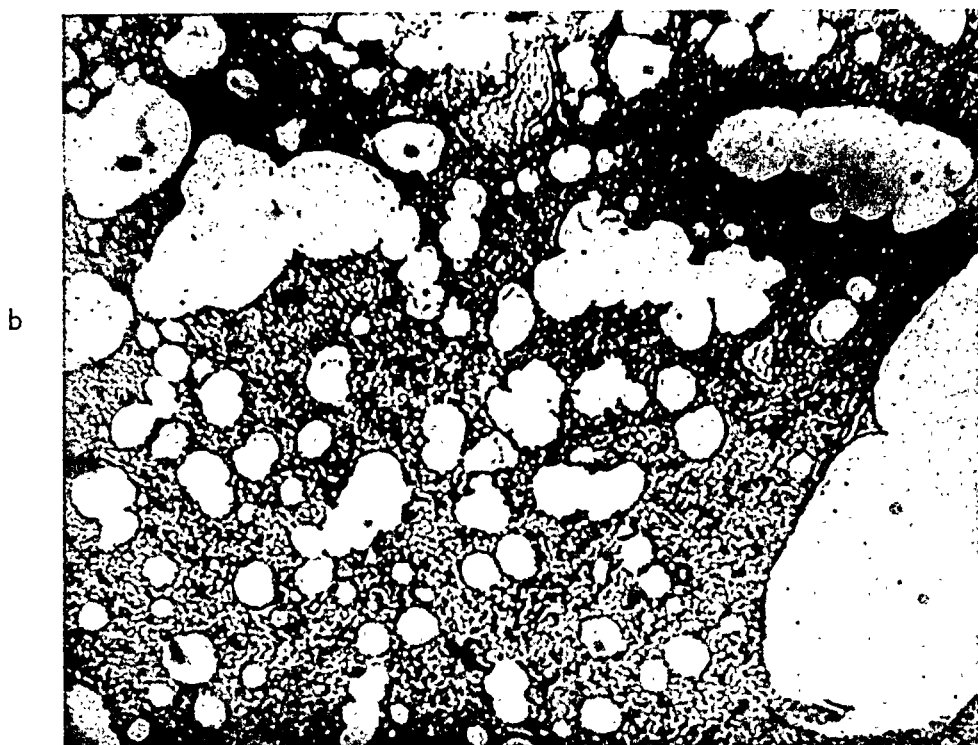


Fig. 41. Rabbit K 17. *a*. Myocardium with broken muscle bundles and haemorrhages. *b*. Lung with haemorrhages and emphysema. Hematoxylin — van Gieson.  $\times 78$ .

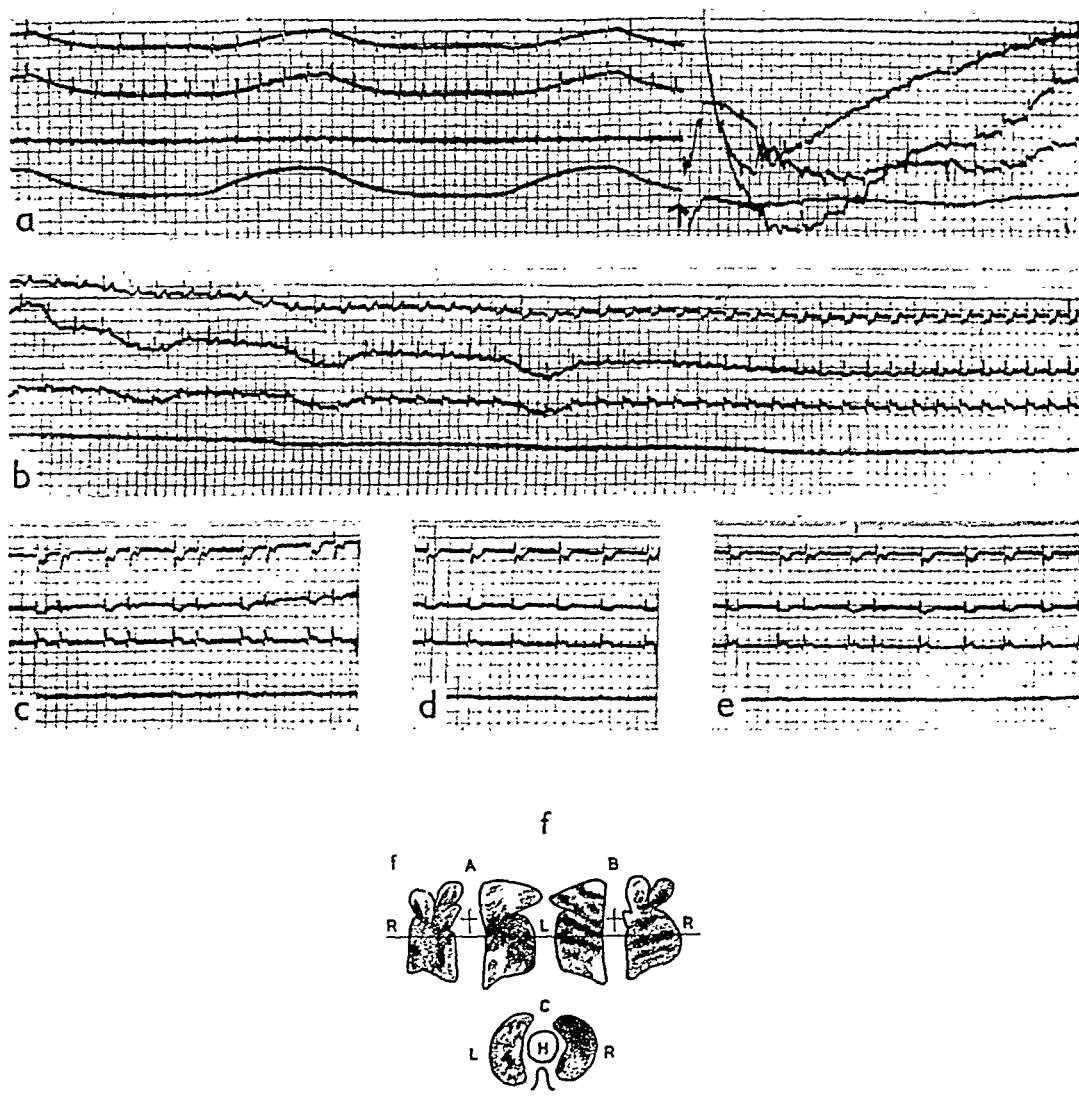


Fig. 42 a. Electrocardiogram and respiration of rabbit K 41, exposed after *bilateral vagotomy* to a max. blast pressure of  $19.2 \text{ kgf/cm}^2$  and an impulse of  $26 \text{ gf} \cdot \text{sec/cm}^2$ . a. Before and until 4 sec after the detonation (detonation at arrow). b. 15—26 sec, c. 1.5, d. 2.5 min and e. 2 min 45 sec after the detonation. Time marking 0.1 sec. f. Lungs with haemorrhages.

changes (see fig. 40) indicating severe myocardial lesion (see fig. 41). In this animal a terminal, paroxysmal ventricular fibrillation occurs.

Exceedingly remarkable changes are observed in the *electrocardiogram* of animal K 41 (see fig. 42) exposed to a violent detonation with a maximum pressure of no less than  $19.2 \text{ kgf/cm}^2$  and an impulse of  $26 \text{ gf} \cdot \text{sec/cm}^2$ . In this animal there is a fairly pronounced bradycardia with a frequency minimum of 190/min between 6 and 12 seconds

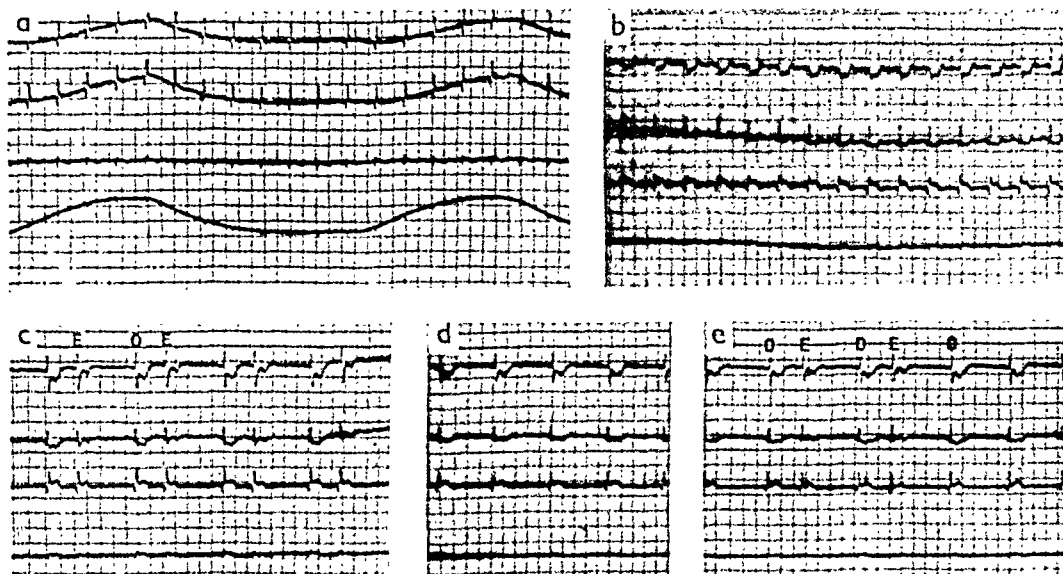


Fig. 42 b. Details of the curves shown in fig. 42 a. *a.* Before detonation. *b.* 20—24 sec, *c.* 1.5, *d.* 2.5 min and *e.* 2 min 45 sec after the detonation. *O* = ordinary RS-complexes. *E* = extraordinary RS-complexes.

against 280/min before the detonation. Other changes are shown in the tabular description below:

*Electrocardiogram before the detonation:* heart rate 280, left axis deviation dependent on the position of the heart.  $P_{III}$  as well as  $T_{III}$  are neg.

After the detonation:

Time after  
detonation Heart rate

0—3 sec 230

3—6 230

6—9 190

9—12 190

12—15 220

15—18 230

#### Changes in the E. C. G.

The rhythm is irregular. Out of the 13 heart beats appearing during this period at least 6 are extrasystoles of different origin. Here is thus a momentary extrasystolia of heterogenic type. Already 1 sec later, further changes appear, suggesting, without doubt, considerable myocardiac lesion.

The curve shows still more noticeable changes, the S—T segment is elevated in leads II and III.

$SI$  now shows a distinct tendency to be depressed.  $S_{II}$  is neutral and  $S_{III}$  retains its previous appearance, is curved and has a high take-off.  $T_{III}$  is neg.

Time after detonation	Heart rate	Changes in the E. C. G.
18—21		S—T more and more depressed.
21—24	240	S—T <sub>I</sub> deeply depressed; increasing depression of S—T <sub>III</sub> .
1½ min	There is now an auricular rate of 240. The impulse from the sino-auricular node appears to take two different paths (see fig. 42 b) one of which is the normal path (the beats marked by O). The continuation of the curve shows that these are the normal beats. In addition to these there is also an extra rhythm (the beats marked by E). Also this rhythm is undoubtedly originated from the sinoauricular node. Its P waves are distinct and of the same shape as those of the ordinary rhythm. The P wave following on the extra beat (E) is not succeeded by a ventricular complex; the line is blocked for the sake of the O-beat. Here there is thus an <i>interference dissociation</i> with regular omission of every third ventricular complex which is an ordinary complex blocked by the extra beat (the E-complex).	
2½ min	The auricular rate is 240, the ventricular rate 120. Here is thus a 2:1 AV block. The extra complexes seen earlier have disappeared. The S—T changes are now still more marked indicating an anterior infarct.	
3	Now there are once more a few E-complexes at the beginning of the curve in consequence of which the subsequent O-complexes are blocked as before. This soon ceases, however, and only the O-complex rhythm appears with a 2:1 AV block.	

The heart stopped approximately 4 minutes after the detonation.

### Discussion.

Heart action is very much less sensitive to blast effects than is respiration. Blast waves with low maximum pressures and small impulses have no effect whatever upon the heart. A blast wave with high pressure, on the other hand, causes considerable changes in the heart rate, the coordination and in the size and shape of the different complexes.

The first and most constant sign of blast effects on the heart function is a reduction of the heart rate. This bradycardia begins immediately after the detonation and has generally reached its maximum already within 10 sec. In animals exposed to severe blast effects the rate after the detonation is only one tenth to one twentieth of the initial value. This great reduction, however, is of short duration, and even in the most prominent cases the rate has again increased af-

ter 20 or 30 seconds. In animals which survive, however, the heart rate often remains reduced for several hours after the detonation.

Even after the most violent detonation there is always, before the animal dies, a certain rate increase after the initial bradycardia. This increase of the heart rate may be the result of a liberation of *adrenaline*, but may also be a reflex action caused by a raised pressure in the great veins and the right ventricle (the Bainbridge reflex). *In no case has the heart action ceased at once after the detonation.*

KROHN *et coll.* (1942) state that the bradycardia caused by the detonation also appeared if the animals had been vagotomized or atropinized. *The present investigations show, however, that the slowing-up of the heart action is dependent on intact vagi. In animals, which have been vagotomized before being exposed to the detonation, no change or only slight change of the heart rate is seen.* The result is the same after bilateral denervation of the carotid sinus. In animals, on the other hand, which have been sinus denervated but not vagotomized before the experiment, a bradycardia of about the same extent as in the untreated animals sets in after the detonation. It may be pointed out, however, that in addition to denervation of the carotid sinuses also bilateral vagotomy is necessary in order to interrupt entirely the carotico-aortic depressor afferents.

A bradycardia may be caused in several ways. An intracranial pressure increase leads to bradycardia because of stimulation of the vagus centre. The detonation causes a momentary increase of the cerebrospinal fluid pressure, as already mentioned, which, however, is of very short duration. HEYMANS (1928) found that the bradycardia, caused by a temporary increase of intracranial pressure, disappears already a few seconds after the increase has ceased. It seems improbable, therefore, that the bradycardia caused by the detonation and which may remain for quite a long time, would be a result of such a pressure rise. It may also be pointed out here, that in animals whose heads have been exposed to violent shock waves, no bradycardia occurs if their bodies have been protected and they have got no pulmonary or abdominal injuries.

The experiments with vagotomized and sinus denervated animals prove that the effect on the heart rate is of reflex origin and that vagus is the main afferent path.

Such reflexes may be caused by stimulation of different regions of the organism.



HERING (1923, 1927) has shown that pressure against the sinus caroticus region causes reduction of blood-pressure and bradycardia. As bradycardia, however, also occurs in animals which have been sinus denervated this mechanism seems to play no decisive part here, a fact which also may be concluded from some experiments made by v. SAALFELD (1932).

As bradycardia seems to be connected in some way with the extent of lung lesion it is probable that stimulation of the vagus endings in the lung, mentioned in the previous chapter, play the main part in producing these reflexes. This supposition is also supported by earlier observations. SCHWIEGK (1935) found, thus, that an increase of the static pressure in the lung caused reflexive bradycardia and reduction of the systemic blood-pressure. Both these effects would be dependent on intact vagus function. v. SAALFELD (1932) found that expansion of the lung causes reflexive slowing of the heart action and is of opinion that it is produced by stimulation of pleural stretch receptors. Also CAPPS and LEVIS (1907) have observed vagal reflexes produced by irritation of pleura. The supposition that bradycardia is produced by distension of the lung and not by central influence is also supported by the fact that a sufficiently rapid drop in atmospheric pressure (explosive decompression) produces bradycardia and reduction of blood-pressure, but only slight change of the cerebrospinal fluid pressure (WHITEHORN, LEIN, EDELMAN and HITCHCOCK 1947).

Vagal bradycardia can also be produced by stimuli to other organs, a fact possibly of some importance in this connection. Thus, vagal reflexes can be elicited in, for instance, the upper respiratory passages, particularly from the larynx and also from the eye balls and from abdominal viscera (cf. WHITEHORN, EDELMAN and HITCHCOCK 1946).

The possibility of an effect of the shock wave direct on the heart (pressure- or contusion effect) must also be considered. After a contusion of the heart, however, there is usually an immediate acceleration of the heart rate (BRIGHT and BECK, 1934/35), while bradycardia, on the other hand, would be more rare (MORITZ and ATKINS 1938).

In addition to the initial bradycardia there are a number of different changes in the electrocardiograms from animals exposed to blast waves with high maximum pressures. Rhythm changes are common, often of the sinus arrhythmia type, occasionally increasing to complete irregularity. Extrasystoles of different origin are common, and bigeminy may occur.

Characteristic for the electrocardiograms from blasted animals seem to be the rapid changes in size and shape of the different complexes, and that they change rapidly from positive to negative, or vice versa. The changes are, in many ways, similar to those in myocardiac injury and heart infarct. Microscopical examination of the heart muscles often shows severe lesions of the muscle fibres and haemorrhages between these. The sudden E. C. G. changes would, at any rate, be due partly to the haemorrhages. Another important factor may be the altered position of the heart and the direction of its electrical axis due to the extensive pulmonary haemorrhages.

Possibly a number of symptoms, for instance, the reduction of blood-pressure, to be described later, could, partly at any rate, be due to reflex action from the severely injured heart muscle itself, (cf. JARISCH and RICHTER 1939, JARISCH 1941 and DIETRICH and SCHMIERT 1939). In the most severe cases the monophasic curves, typical of the dying heart, are often seen immediately after the detonation. These often change, however, before the animals die, to return again just before death.

The heart dies usually during an increasing auriculoventricular blocking. In the early stages the auriculoventricular conduction is generally normal or somewhat lengthened, later it is further lengthened.

*Ventricular flutter or fibrillation occur but does not seem common, and they appear relatively late.* In one case only, a vagotomized animal, a ventricular flutter did appear already after 7.7 seconds. This ceased, however, already after 4.6 seconds and was succeeded by a change of the right bundle branch block type.

Part of the E. C. G. changes, besides the bradycardia, may possibly be due to a high vagus tonus (see e.g. NORDENFELT 1941), but for the main part they must be considered due to the lung haemorrhages and the direct contusion effect against the heart when the chest is struck by the blast wave.

Changes in the heart caused by contusion of thorax have been known for a long time. A number of different changes have been described e.g. *extrasystoles* (KAHN and KAHN 1928, MORITZ and ATKINS 1938), *heart block* (COFFEN 1930, WALKER 1937, FUNK 1945) *auricular flutter* (BARBER 1938) *myocardiac lesion with infarct electrocardiograms* (ANDERSON 1940) and *ventricular fibrillation* (BRIGHT and BECK 1934—35, MORITZ and ATKINS 1938). MORITZ and ATKINS have

even observed instant *asystolia* after contusion direct against the heart.

Other E. C. G. changes occurring in heart contusion (BRIGHT and BECK 1934—35, ANDERSON 1940) are abnormalities of the T wave which may be large or inverted. The Q wave is often abnormally deep. Slurring and notching of the QRS complexes are common. High or low take-off of the S-T-interval may be seen.

The heart may rupture after trauma but this does not seem particularly common (KELLERT 1917, TUOHY and BERDEZ 1926, CLOVE, KELLERT and GORHAM 1934, BRIGHT and BECK 1934—35). One case of rupture of the left ventricle caused by a dynamite explosion has been described by MILLER (1947). In the present material no case of heart rupture has been found, but on the other hand, there was in one animal an intense aneurysmatic dilatation of the right ventricle. A moderate right dilatation is common in animals with severe lung lesions.

*The experiments described in this chapter would lead to the following conclusions:*

*The heart action, though not so sensitive as respiration, may be highly affected by blast waves with high max. pressures.*

*The most prominent result would be bradycardia which sets in immediately after the detonation and which in severely injured animals may reach quite excessive degrees. The bradycardia seems to be caused by reflexes chiefly originating from the damaged lungs. It is dependent on an intact vagus function. A shock wave acting upon the head does not give rise to bradycardia if the thorax and abdomen have been protected and there are no lung injuries. In such cases there is instead an acceleration of the heart action probably due to liberation of adrenaline. Even in the most severe blast injuries the heart rate is increased again after the initial bradycardia and no animal has died of an instantaneous cardiac standstill.*

*After violent detonations, severe changes are seen in the electrocardiogram, indicating severe myocardial lesion. A direct contusion of the heart caused by the impact of the blast wave on thorax seems to play an important role. The myocardial lesions have been verified microscopically.*

*Sinus arrhythmia, sometimes reaching as far as to complete irregularisation of the rhythm, is common. Extrasystoles of different origin oc-*

*cur, and bigeminy may be seen. An interference dissociation occurred in one case. Different types of heart block may be found, and the heart usually dies with an increasing auriculoventricular blocking. Ventricular flutter or fibrillation may occur but does not seem to be common, and they are a late occurrence.*

*The E. C. G. complexes often change their shape and charge very rapidly.*

*No rupture of the heart or of the great blood vessels have been seen, but a moderate dilatation of the right ventricle is a common feature in animals which have lived for some time after a violent detonation.*

## CHAPTER 14.

### Effects on the Peripheral Circulation.

#### 1. Arterial blood-pressure.

MOTT (1916), already, states that circulatory changes are common in "shell shock", and that the arterial blood-pressure is often below 110 mm and never above 135 mm Hg.

BARROW and RHOADS (1944) found in people injured by blast a systolic blood-pressure of 80—90 mm Hg and a diastolic pressure of 40—50 mm Hg. This hypotonia reacted promptly to intravenous administration of plasma.

Most clinical observations seem to show, however, that the blood-pressure is usually normal or rather raised in blast injury. Thus TUNBRIDGE and WILSON (1943) mention that the blood-pressure in people injured by blast and examined one to three hours after the accident, was raised until approximately one hour before death in uncomplicated cases, in spite of obvious signs of pulmonary congestion and tachycardia. A lowered arterial blood-pressure (systolic pressure  $< 100$  mm Hg) did not exist unless the blast injury was combined with other injuries like burns or extensive wounds accompanied by serious surgical shock, and if the patient was moribund. The initial, systolic blood-pressure of those injured (men in the ages 19—38) varied between 100 and 160 mm Hg, and the diastolic pressure between 60 and 68 mm, except in one case where it was 100 mm Hg. KLEMM (1945) examined 35 persons exposed to blast waves. No significant changes in the blood-pressure existed. They were, however, examined first several days after the detonation. LEAVELL (1945) found in a number of soldiers injured by blast one or two cases with raised venous pressure (22.5 cm of water) but otherwise hardly any abnormal blood-pressure values were noticed.

Finally, RUSKIN, BEARD and SHAFFER (1948), who have examined 180 casualties from the explosion catastrophe in Texas City in 1947, found that most of the injured had a diastolic hypertonia when examined already one hour after the injury. Many of their patients had a systolic blood-pressure of 210—230 and a diastolic pressure of 140—160 mm Hg.

Blood-pressure examinations in blasted animals have been published only by HOOKER (1924), and KROHN *et coll.* (1942).

HOOKER was able to observe a rapid reduction of the arterial blood-pressure in dogs exposed to the pressure field around a gun muzzle. The mean arterial blood-pressure in the femoral artery was obtained by means of a mercury manometer. Seven minutes after the explosion the blood-pressure in one animal had fallen from 150 to 50 mm Hg at which value it remained until immediately before the animal died 4 hours later. In one animal in chloral anaesthesia the pressure had fallen from an initial value of 84 to 36 mm Hg 10 minutes after the detonation. It remained at this value for about one hour, after which it again fell slowly until the animal finally died 10½ hours after the firing.

In blast experiments with high explosives the blood-pressure fell only in one animal out of five. The arterial pressure then dropped from 114 to 74 mm Hg, and the venous pressure from 8.75 to 6.25 cm of water.

The venous pressure in the femoral vein was measured with a water manometer as soon as possible after the detonation (usually after about 7 minutes). In five animals which were not shocked<sup>1</sup>, the venous pressure fell in two of the animals, rose in two, and remained unchanged in one. In five animals with partial shock it fell in two and rose in three, and finally in six animals completely shocked, the venous pressure fell in four cases and rose in two. He considers this to prove that the venous pressure is lowered in air concussion shock, and is of opinion that the reduction cannot be secondary to a decreasing arterial pressure. No relation could be found between the reduction of blood-pressure and the extent of lung injury.

KROHN, WHITTERIDGE and ZUCKERMAN measured the arterial blood-pressure in 10 rabbits after the blast, both by the bloody method with a cannula in the carotid or femoral artery, and by VAN LEERSUM's (1912) carotid loop method (in 4 animals). *Even in the*

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<sup>1</sup> Hooker means by shock a sudden reduction of arterial blood-pressure to approximately one half of the initial value.

*most favourable cases, however, values could not be obtained earlier than about 4 minutes after the explosion.*

At the maximum pressures used by these authors (half to twice the  $P_{50}$  value) there was always a fall in blood-pressure. It was related to the maximum blast pressure obtained.

In animals with such severe injury that death followed within a few minutes, no blood-pressure recordings could be made, but the pulse in one animal was palpable up to 30 sec after the detonation. In animals which did not die at once but had obtained such severe injuries, nevertheless, that they died within half an hour after the detonation, the blood-pressure had fallen from approximately 90 mm to 30—40 mm Hg at the first recording. It then remained at this low level until the animal died.

Animals which survived the detonation in spite of relatively severe internal injuries, showed an immediate reduction of blood-pressure. In the course of the next hour the pressure rose again slowly up to normal. In one case the pulse was not palpable during the next 4 min after the explosion, but after this the blood-pressure rose rapidly and after 10 min it was normal again. Finally, in animals with only a minor transient reduction of blood-pressure, it had reached its normal value again after 30 minutes or less.

In discussing the causes of the blood-pressure fall the authors point out that they have not observed any signs of vagal slowing-up of the heart action except in animals with lesions on cervical vertebrae. They maintain further, that there was an immediate reduction of the blood-pressure in animals with either thorax or abdomen entirely protected by a close-fitting plaster jacket. They state, therefore, that "it is evident that either pulmonary or extrapulmonary trauma can cause an immediate drop in blood-pressure and clearly both factors may operate in an unprotected animal exposed to blast".

#### **Own investigations.**

From the experiments mentioned previously it is clear that quite a number of radical changes in the heart action can be caused by the blast wave. Because of this, it seems probable also that changes of the peripheral blood-pressure would constitute an important part of the blast effects upon the circulation.

TABLE 29. Max. pressure and impulse values, blood-pressure and quotients of lung injury in rabbits, the blood-pressure of which has been recorded with the bloody method.

Rabbit	Max- pressure kgf/cm <sup>2</sup>	Impulso gf · sec/cm <sup>2</sup>	Blood-pressure in left common carotid artery				Quotients of lung injury				Comments
			Before detona- tion	After detona- tion (max. decrease)	Per cent	Time after detonation, when this value has been recorded	Haemorrhage (+ oedema)		Increase of lung volume (emlysema)		
							Left lung	Right lung	Left lung	Right lung	
K 43	5.1	5.4	90	80	11	0.5 sec	1.11	1.06	1.00	0.93	Died after 20 min
K 35	6.7	7.6	85	65	24	6 »	1.26	1.31	1.08	1.17	
K 33	9.2	12.8	120	70	42	5 »	moderate	severe	—	—	
K 39	9.7	11.3	95	40	50	10 »	1.21	1.84	0.96	1.09	Died within one min
K 27	11.9	20.8	120	75	38	10 »	1.33	1.74	1.12	1.25	
K 32	11.9	20.5	60	0	100	12 »	1.64	2.06	0.88	0.88	
K 36	12.5	11.9	75	50	33	5 »	1.02	1.20	1.17	1.27	Died within a few min
K 31	13.3	23.9	75	0	100	60 »	1.20	1.36	1.01	1.01	
K 40	13.3	17.0	115	0	100	11 min	2.77	3.16	1.53	1.65	

The purpose of the blood-pressure studies has been partly to gain information on the changes occurring *immediately after the detonation* and while the bradycardia is most pronounced (i. e. the two or three minutes immediately after the detonation), and partly also to follow the blood-pressure during a longer period (some 48 hours) in order to observe a possible drop in blood-pressure as a sign of traumatic shock. The changes first mentioned appear to be of great interest, but have in spite of this not been studied earlier.

#### a. Changes in blood-pressure immediately after the detonation.

Recordings of the blood-pressure changes which occur immediately after the detonation, have been made by using the bloody method, described on page 33. Owing to very considerable technical difficulties such recordings have only been possible in blasting experiments in the detonation chamber and only to a limited extent (some 20 animals in all, 11 of which had been bilaterally vagotomized and sinus denervated before the detonation).

Table 29 gives pressure and impulse values and also quotients of lung lesions and data concerning the blood-pressure of the 9 animals with intact vagus and carotid sinus function. They have been grouped in the table according to increasing maximum pressure values.

At the moment of the detonation there is a momentary rise in pressure (see fig. 43). This rise, which only lasts for a fiftieth up to a twentyfifth of a second, is partly due to a direct effect of the blast wave on the transmission tube between the animal and the recording apparatus, but it is principally due to a momentary, intravasal pressure rise caused by the detonation. This brief pressure rise is usually quite considerable, but its absolute value has not been determined with certainty except in one case where the increase was insignificant (5 to 10 mm Hg) in spite of the fact that the maximum pressure of the blast wave was 11.9 kgf/cm<sup>2</sup> and the impulse 20.5 gf·sec/cm<sup>2</sup>.

Immediately after this initial pressure peak the blood-pressure, as shown in the figures, is usually about 10 to 20 mm higher than the value immediately before the detonation. In some animals (K 27, K 31 and K 32), however, the pressure immediately after the detonation is lower than before.



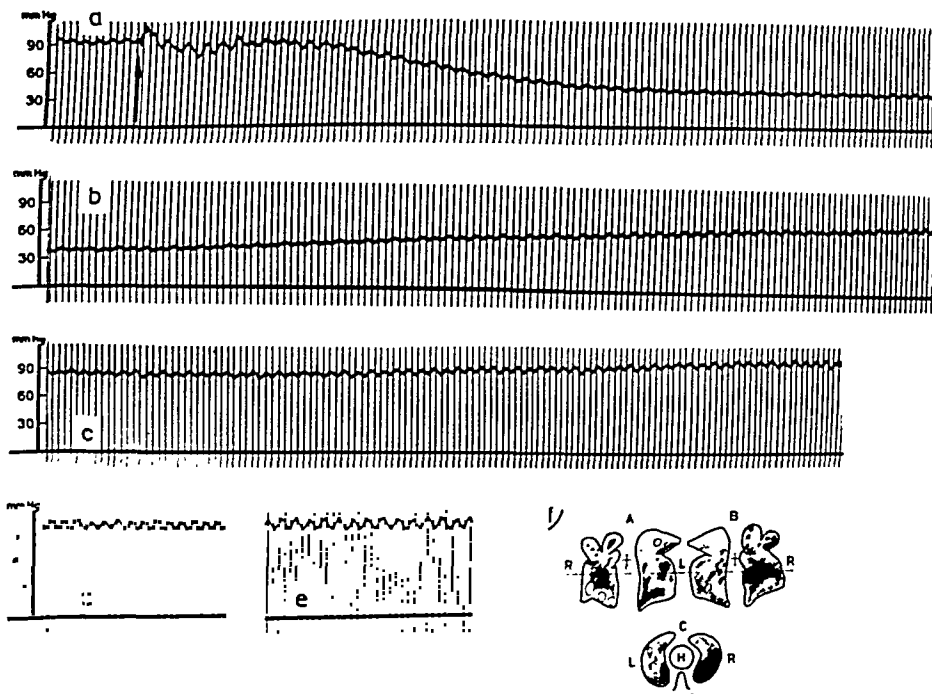


Fig. 43. Blood-pressure in left common carotid artery. Rabbit K 39, exposed to a max. blast pressure of 9.7 kgf/cm<sup>2</sup> and an impulse of 11.3 gf·sec/cm<sup>2</sup>. a: Before and until 12 sec after the detonation (detonation at arrow). b. 12—25 sec, c. 50—60 sec, d. 4 min and e. 6 min after the detonation. Time marking 0.1 sec. f. Lungs with haemorrhages and bullous emphysema.

*The blood-pressure begins to fall almost immediately after the detonation, and in the cases where the animal survives, it is generally at its lowest 5—10 seconds after the detonation (see fig. 43). The extent to which the blood-pressure drops, as shown in table 29, is in some way related to the violence of the detonation and consequently also to the extent of the lung lesion. The duration of the blood-pressure fall also appears to depend on the force of the detonation. Thus in rabbit K 43, exposed to the smallest pressure and impulse effect obtained in this group the blood-pressure, after a slight drop, reached its original value already after 1½ sec. In rabbit K 27, on the other hand, the blood-pressure did not reach its original value even 5 minutes after the detonation.*

In cases where the animal dies in connection with the detonation, the blood-pressure falls fairly rapidly down to zero, and the electrocardiogram shows that the heart action is ceasing (e. g. rabbit K 40, fig. 44). A fairly singular phenomenon was observed in one animal (K 32). The blood-pressure dropped rapidly almost down to zero

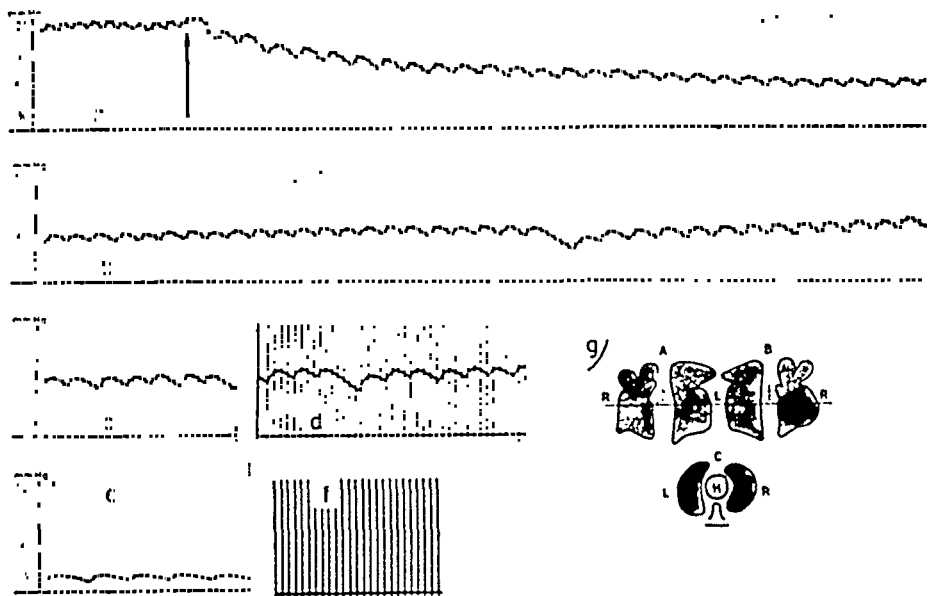


Fig. 44. Blood-pressure in left common carotid artery. Rabbit K 40, exposed to a max. blast pressure of  $13.3 \text{ kgf/cm}^2$  and an impulse of  $17.0 \text{ gf} \cdot \text{sec/cm}^2$ . *a.* Before and until 11 sec after the detonation (detonation at arrow). *b.* 11—23 sec, *c.* 2, *d.* 3, *e.* 10 and *f.* 11 min after the detonation. Time marking 0.1 sec. *g.* Lungs with haemorrhages.

after the detonation, and remained there with slight fluctuations for almost 5 minutes. After this there was an increase which lasted less than one minute, however, after which the pressure once more dropped to zero without the original value having been reached.

Immediately after the detonation some more or less strong fluctuations appear in the blood-pressure suggesting a severe strain on the regulatory mechanism. These fluctuations are specially marked in rabbit K 31 (fig. 45). In this animal the arterial pressure dropped from about 75 mm down to 15 or 20 mm Hg within 0.1 sec after the detonation, and rose again to approximately 90 mm Hg within a further 0.1 sec. This was followed by another drop to about 20 mm Hg during the course of 0.2 sec and then by a further rise up to the initial value. It was then stabilized but soon began to fall again slowly, reaching zero at approximately one minute after the detonation. In rabbit K 32 a sudden rise in blood-pressure of about 15 mm Hg was seen 0.2 sec after the detonation.

In animals which survived the detonation, the arterial pressure, after the initial drop, was of about the same value as before the detonation, or even 10—15 mm Hg higher.

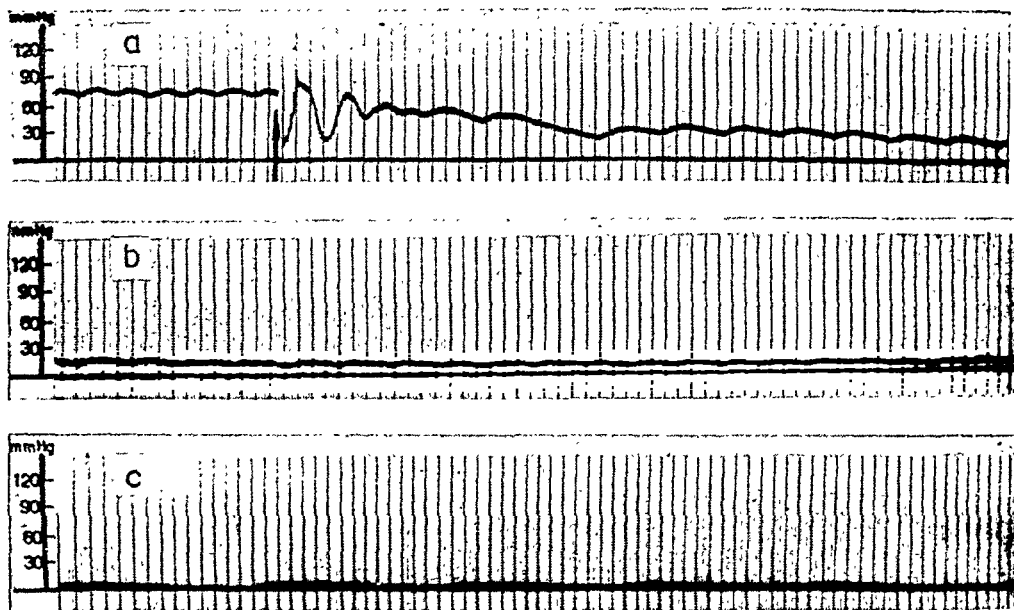


Fig. 45. Blood-pressure in left common carotid artery. Rabbit K 31, exposed to a max. blast pressure of  $13.3 \text{ kgf/cm}^2$  and an impulse of  $23.9 \text{ gf} \cdot \text{sec/cm}^2$ . a. Before and until 5.5 sec after the detonation (the detonation occurred just at the arrow). b. 7.5—14 sec and c. 1 min after the detonation. Time marking 0.1 sec.

### b. Changes in blood-pressure after the development of blast injury.

When the purpose has been to observe the variations of blood-pressure for a longer period of time, the bloody method used in the experiments mentioned above, was not suitable. Therefore, the bloodless method for measuring the pressure in the central artery, described on page 34 above, has been used instead. All estimations have been made on unanaesthetized rabbits. The first determination on each animal has generally given higher and more varying values than those following, as the animal was restless at the beginning. These values have been discarded. After a few series of determinations, however, the animals have become accustomed to the apparatus, and the values obtained in one and the same series, are more regular.

The blood-pressure values obtained by this method vary within quite broad limits in different rabbits and are considerably lower throughout than those obtained by the bloody method. In determinations on a material consisting of 50 normal rabbits with a body weight varying from 1.5 to 3.5 kg the present author thus found

values between 41.6 and 88.0 mm Hg with a mean value of  $59.9 \pm 1.8$  mm Hg ( $\sigma = 12.7$  mm Hg).

These values are lower than most of those stated in the literature. By a similar method ANDERSON (1922—23) thus obtained 76—87 mm, WATSON (1926) 75—90 mm, SQUIER (1927—28) 50—74 mm, SCHMIDT-WEYLAND (1931—32) 80—90 mm, GRANT and ROTHSCHILD (1934) 70—90 mm, and LITHANDER (1945) a mean value of the blood-pressure of 65 mm Hg.

Lower values, however, have been obtained with a similar method by, for instance, KURAYA (1923), namely 35—50 mm Hg. He states that the pressure in the central artery of the ear is always 56—63 mm lower than in the carotid artery. According to BEHRENS (1926) the mean value of the pressure in the central artery of the ear is about 40 mm Hg, and the values are fairly constant for one and the same animal. Finally, it should be mentioned that FAHR (1938—39), whose method the present author has used with certain modifications, has obtained values of between 40 and 80 mm Hg, thus corresponding well with the results in the present investigation.

The main reason for the variations in the results obtained by the different authors is probably to be found in differences in the methods used. The variations in different rabbits may depend on various reasons, for instance, the age and weight of the rabbit (ANDERSON 1922—23). Body movements increase the blood pressure (ANDERSON) and also excitation (v. RECKLINGHAUSEN 1906, VAN EWYK and SCHMIDTMANN 1921—22, KURAYA 1923, BECKMAN 1925—26, SQUIER 1927—28, GRANT and ROTHSCHILD 1934, DOWNMAN *et coll.* 1944, LITHANDER, and others). This factor in addition to an insufficient dilatation of the artery of the ear (v. RECKLINGHAUSEN, ANDERSON, KURAYA, BEHRENS, FAHR, DOWNMAN *et coll.* LITHANDER and others) is the main cause of the variations and the chief source of the errors.

The pressure in the carotid artery, measured by the bloody method, is generally considered to be between 80 and 110 mm Hg (see WOODBURY and HAMILTON 1937, LITHANDER 1945, and others), and in this material it lies between 75 and 120 mm Hg. The differences in the two methods, therefore, are quite considerable, and BOGOMOLEZ (1911) points out that changes in pressure in the central and peripheral arteries do not always run parallel. GRANT and ROTHSCHILD (1934) however, on the basis of their careful studies, believe that the systolic blood-pressure in the artery of the ear, measured under such conditions as prevail in determinations of this kind, is a reliable index of the systemic blood-pressure.

As the changes of the blood-pressure in the present observations have been examined at different times before and during some 48 hours after the detonation, it is important to get an idea of the variations of blood-pressure in normal rabbits during a 24 hours period. For

TABLE 30. Mean variation of blood-pressure during one day.

Time for measurement	<i>n</i>	$M \pm \varepsilon(M)$	$\sigma$
9 o'clock .....	27	$49.0 \pm 0.97$	5.05
13 » .....	27	$49.0 \pm 1.19$	6.17
15 » .....	23	$48.8 \pm 1.13$	5.44

this purpose the blood-pressure has been measured in a number of animals on one and the same day at 9 and 13 and 15 o'clock. The mean values in animals with a body weight from 1.5 to 2.5 kg and their standard errors and deviations are found in table 30.

These mean values are lower than those obtained above, probably because these animals have had a somewhat lower average body weight. No statistical difference exists between the blood-pressure values ( $P > 0.9$ ) measured at these three different times of the day.

In addition to this the variations in blood-pressure have been examined during a period of 48 hours. See table 31.

A certain slight fall in blood pressure is seen here. There is no statistical difference, however, either between the values obtained at the same time on different days ( $0.9 > P > 0.5$ ) or between the values obtained at different times ( $0.9 > P > 0.3$ ).

In the animals injured by blast the blood-pressure has been measured at different times during the days immediately before the experiment as well as just before the experiment. After the detonation another determination has been made as soon as possible (usually 5—10 minutes after), and then generally 1, 2, 4, 6, 24 and 48 hours after the detonation.

TABLE 31. Mean variation of blood-pressure during two consecutive days.

Time for measurement	$n$	$M \pm \varepsilon(M)$	$\sigma$	
9 o'clock {	first day	17	$48.2 \pm 1.27$	5.25
	second day	16	$47.0 \pm 1.43$	5.73
13 o'clock {	first day	17	$47.9 \pm 1.50$	6.19
	second day	16	$47.3 \pm 1.52$	6.05
15 o'clock {	first day	13	$46.4 \pm 1.62$	5.84
	second day	15	$46.8 \pm 1.38$	5.16

TABLE 32. Variations in arterial blood-pressure of rabbits with slight lung injuries (quotient of haemorrhage for right lung  $< 1.50$ ). The values are given in per cent of the blood-pressure value obtained immediately before the detonation.

Time after detonation	Animals injured by blast				Normal rabbits	
	Rabbits not bled		Bled rabbits			
	<i>n</i>	$M \pm \varepsilon(\dot{M})$ per cent	<i>n</i>	$M \pm \varepsilon(M)$ per cent	<i>n</i>	$M \pm \varepsilon(M)$ per cent
5—10 min	7	— 4.3±4.62	10	—14.5±6.76	25	+0.2±2.15
1 hour	4	—17.7±9.26	7	—16.9±4.03		
2 hours	5	— 9.4±6.24	9	—13.6±4.82		
4 »	5	—12.3±5.14	8	—15.1±4.44	24	—0.5±1.85
6 »	4	— 5.2±8.49	7	—13.7±4.13		
24 »	6	— 4.6±2.31	10	—14.7±3.53	25	—1.6±2.14
48 »	4	— 9.1±2.59	6	—15.0±6.00	15	—1.8±2.15

The material consists of 25 animals with severe lung lesions (quotient of haemorrhage for right lung  $> 1.50$ ) and 17 with slight lesions (quotient of haemorrhage  $< 1.50$ ). From a number of these animals, namely, 15 of those severely injured and 10 of those with slight lung lesion, blood has also been taken for chemical blood analysis (see page 174 below). The samples (5 or 7 ml each time) have been taken in connection with the blood-pressure determination, but always after this.

As control material for the statistical calculation, 25 normal rabbits have been used in which the blood-pressure has been measured at about the same times as in the experimental animals.

When studying the results the animals from which blood samples have been taken have been dealt with separately owing to the possible changes of the blood-pressure which may have been caused by the blood loss.

The results of the experiments are seen in tables 32 and 33 (severely and slightly injured animals, respectively), which include the mean values of the percental change of the blood-pressure at different times compared to the value obtained immediately before the detonation. The reason why the blood-pressure obtained immediately before the detonation has been chosen as initial value, and not a mean value of all determinations made on one and the same animal before the

TABLE 33. Variations in arterial blood-pressure of rabbits with severe lung injuries (quotient of haemorrhage for right lung  $>1.50$ ). The values are given in per cent of the blood-pressure value obtained immediately before the detonation.

Time after detonation	Animals injured by blast				Normal rabbits	
	Rabbits not bled		Bled rabbits			
	<i>n</i>	$M \pm \varepsilon(M)$ per cent	<i>n</i>	$M \pm \varepsilon(M)$ per cent	<i>n</i>	$M \pm \varepsilon(M)$ per cent
5—10 min	10	+ 1.3 ± 7.54	15	— 9.1 ± 4.79	25	+ 0.2 ± 2.15
1 hour	9	— 12.9 ± 10.17	15	— 21.2 ± 3.65		
2 hours	8	— 13.3 ± 8.65	14	— 21.0 ± 4.48		
4 »	8	— 17.9 ± 6.87	14	— 22.6 ± 5.22	24	— 0.5 ± 1.85
6 »	8	— 14.6 ± 3.28	13	— 14.6 ± 4.76		
24 »	8	— 5.1 ± 8.97	11	— 13.3 ± 4.19		
48 »	3	— 6.2 ± 16.92	10	— 4.0 ± 6.53	15	— 1.8 ± 2.15

detonation, is because the first-mentioned value has been considered the most accurate, due to the animals having had time to get accustomed to the procedure. Furthermore, there is, as shown previously, no statistical difference between the values determined at different times of the day.

It is evident that the blood-pressure is usually lower in the animals injured by blast than in the normal animals. The variations are extremely great, however, a fact shown by the great standard errors of the mean values (see tables). In the statistical study all values from each group with similar extent of injury have been added up irrespective of the time when the determinations were performed, and the result has been compared with the values obtained from the control animals. The difference is statistically significant both in regard to the slightly injured animals and those with severe injury ( $P < 0.001$ ). A comparison between blood-pressure changes in animals from which blood samples have been taken and in animals which have not been bled, shows that the blood-pressure is generally, though not throughout, lower in the first-mentioned group. This difference is statistically probable as regards the group of slightly injured animals ( $0.05 > P > 0.02$ ). On the other hand, there is no significant difference as to the group of severely injured animals ( $0.2 > P > 0.1$ ).

low values (30 or 40 mm Hg) and remains at this level with slight fluctuations until the animal dies, or is killed. The secondary increase of blood-pressure, which was noticed in the surviving non-treated animals and in the vagotomized animals has not been observed in animals with denervated carotid sinuses.

A certain, though slight, increase of pressure, however, occurred in one animal (rabbit K 71). The pressure after the detonation dropped within 10 seconds from the initial value of 65 mm Hg to 20 mm Hg. Fifteen sec after the detonation the pressure was 10 mm Hg, and after a further five sec it had dropped to zero. It remained at zero until approximately  $1\frac{1}{2}$  min after the detonation, when it rose again to 15 or 20 mm Hg which value was then kept during the next minute.

During the seconds immediately after the detonation the blood-pressure curve was somewhat irregular and had an increased amplitude. It is hard to say whether this was due to an actual increase of the pulse amplitude or to disturbances caused by the swinging of the pendulum after the detonation. In some blasting experiments with the blood-pressure gauge connected to the detonation chamber, but with no animals, some disturbances due to vibrations were obtained immediately after the detonation. Probably the irregularities and the increase of the amplitude in the curve are due both to such disturbances and to a certain actual increase of the pulse amplitude.

### Discussion.

Blood-pressure recordings in blasting experiments have shown that the systemic arterial blood-pressure drops almost immediately after the animal has been struck by the blast wave. The extent and duration of this reduction have a certain relation to the extent of the blast injury, and consequently also to the force of the shock wave. In animals which have been exposed to violent detonations and have obtained severe lesions the fall in blood-pressure may thus reach excessive proportions.

As mentioned already earlier a pressure against the region of the carotid sinus causes a blood-pressure fall which has been shown by HERING (1927) to be due to a direct effect on the carotid sinus. The strong but extremely brief pressure caused by the detonation seems unimportant in this connection, however, a fact also indicated by the



present experiments with sinus denervated animals. The pressure is probably of much too short duration to have an effect on the sinuses. Furthermore, the magnitude of the pressure peak would be of slight significance as the sensitiveness of sinus is greatest when the pressure acting in it is of about the same value as normal blood-pressure (quoted from ASK-UPMARK 1935). On the other hand, the present experiments have shown that the blood-pressure increase which succeeds the blood-pressure fall immediately after the detonation, is dependent on an intact carotid sinus mechanism, as it fails to appear in sinus denervated animals where the pressure remains for a long time at the low level to which it dropped immediately after the detonation, or continues to fall slowly, probably as a result of the progressive lung haemorrhages.

As a cause of the blood-pressure drop, however, greater importance must be assigned to an intrathoracic or intrapulmonal pressure increase. Thus YOUNG (1945), in his experiments mentioned earlier, obtained a fall in arterial blood-pressure "by simulating in slow motion the effects of increased pressure on the animal body". A distension of the lungs by increasing the intratracheal pressure produced an even more marked blood-pressure fall (cf. also POLAK and ADAMS 1932 and TATUM and GINZLER 1946). WHITEHORN, EDELMAN and HITCHCOCK (1946) could produce a drop in blood-pressure in dogs exposed to a sufficiently rapid decompression. Not even in these cases is it likely, that a direct action of pressure against the carotid sinus region has been the cause. The authors consider that the primary reason for the blood-pressure fall in explosive decompression is a reduction of the venous reflux to the heart caused by distension of the lungs and increase of the intra-thoracic pressure produced when the speed of the decompression is greater than that with which air can escape out of the lungs. Reduction of systemic blood-pressure occurred if the speed of the decompression corresponded to a reduction of air pressure  $> 1.4 \text{ kgf/cm}^2$  per sec. In blast decompression is always much more rapid.

KROHN *et coll.* (1942) found that rabbits showed an immediate fall in arterial blood-pressure if lesions occurred in either the thoracic or abdominal organs, by either abdomen or thorax having been protected by a close-fitting plaster jacket. They consider, therefore, that in an unprotected animal both pulmonary and abdominal lesions contribute to the fall in blood-pressure.

The decrease of the heart rate often occurs simultaneously with or even before the blood-pressure fall and must be regarded as the primary phenomenon, while the reduction of blood-pressure seems to be a secondary occurrence and is due to an increase of the pulmonary arterial pressure (KROHN *et coll.* 1942, PARIN 1947) and a reduction of cardiac output. If the fall in systemic blood-pressure were the primary manifestation the heart, when the carotid sinus is intact, should respond instead with an accelerated rate. As a result of the haemorrhages in the lung, and the circulation there having been rendered more difficult, the blood is dammed up in the lungs and the venous reflux to the heart is diminished, which leads to a reduced cardiac output. That this can be a significant reason for a reduction of blood-pressure is confirmed by HOLT, RASHKIND, BERNSTEIN and GREISEN (1946) who have found that the arterial blood-pressure is mainly altered by changes in cardiac output, and only to a lesser degree by changes in the peripheral resistance. Therefore, the decrease of blood-pressure in blast injury should, partly at any rate, be secondary to a vagal slowing-up of the heart, a phenomenon, however, denied by KROHN *et coll.* (1942). Vagal stimulation, as shown by OBERHOLZER (1945) causes a lowering of arterial blood-pressure in the rabbit, but this would not be due to a change of the heart rate.

KROHN *et coll.* (1942) have paid attention to an observation made by several authors (DUNN 1919, BINGER, BOYD and MOORE 1927, GIBBON, HOPKINSON and CHURCHILL 1932, and others), namely, that an increase of the pressure in the pulmonary circulation caused by an obstruction of the pulmonary artery or by a reduction of the total available pulmonary capillary bed, is followed under certain conditions, by a fall in the systemic arterial blood-pressure. At the same time an increase of the pressure in the large veins is observed. Also SCHWIEGK (1935) and DALY *et coll.* (1937) were able to produce an abrupt reduction of the systemic blood-pressure by increasing the pulmonary blood-pressure.

That the lung circulation, by having been rendered more difficult, should be the only cause of a fall in the systemic blood-pressure does not seem probable, however, as a certain reduction of blood-pressure occurs even in animals which have obtained only moderate lung lesions of no greater impediment to the pulmonary circulation. GIBBON *et coll.* (1932) have shown that a reduction of the circulation through the lungs corresponding to a reduction by at least 60 per cent of

the cross-section area of the pulmonary artery, is required in order to produce a significant reduction of systemic blood-pressure as a result of a reduced cardiac output. BRENNER (1935) states that a reduction by at least 75 per cent is required before the systemic blood-pressure begins to drop. — Cf. also v. EULER and LILJESTRAND (1946).

It has been stated by a number of authors (for references see FELDBERG and SCHILF 1930) that the lungs have a noticeably high content of *histamine*. As histamine is liberated in cases of lung injury (cf. e. g. WRIGHT 1948) it seems probable that the changes in blood-pressure may partly be due to histamine action. It has thus been shown by KRULL and HOSOYA and others (quoted from FELDBERG and SCHILF 1930) that histamine in rabbits in deep urethane anaesthesia usually causes a fall in blood-pressure. The present author hopes to be able to discuss further these interesting problems in a future paper.

The most interesting changes in blood-pressure occur during the first 5 minutes after the detonation. In the surviving animals the blood-pressure rises, after the initial drop, up to, or even above, the initial value. The possibility of this secondary rise being dependent on a liberation of *adrenaline* may be considered here. Later the blood-pressure may again fall somewhat, but this decrease is usually not particularly great, and a failure of the circulation is more unusual in animals which have survived the first hour after the detonation. The blood-pressure may remain low for some 48 hours.

## 2. Circulation time.

As far as can be judged from the literature no determinations have been made earlier of the circulation time in blast injury.

The present author has determined the circulation time before and after the detonation in a number of animals exposed in the field (group type B). The determination after the detonation has been made as soon as possible, usually after approximately 30 minutes, and in a few animals also 3 or 4 hours after the detonation.

All determinations have been made in anaesthetized animals.

Twenty animals in all with more or less severe lung lesions have been used, and for comparison also 7 animals with slight lesions. Out of the severely injured animals 9 died before the circulation time could be determined.

In the 11 surviving animals with severe injuries the circulation time (right ear to left eye) before the detonation varied between 2.8 and 6.9 sec with a mean value of  $4.9 \pm 0.4$  sec.

When blast injury has become manifest the circulation time is increased almost in all the rabbits. In only one of the animals it was 0.7 sec shorter after the detonation. The lung haemorrhages of this animal were not particularly severe.

The mean value of the circulation time after the detonation is  $6.2 \pm 0.38$  sec (limit value 3.2 and 7.4 sec). *Thus the average increase amounts to 1.3 seconds or 27 per cent.* This increase is statistically probable ( $0.05 > P > 0.02$ ).

Determinations carried out a longer time after the detonation revealed a further increase of the circulation time. In three animals there was a further increase of 16 %, 44 % and 38 % respectively.

The circulation time in the 7 animals, which had obtained only very slight lung lesions, was before the detonation  $4.8 \pm 0.46$  sec (limit value 3.5 and 6.8 sec). In several of these animals a slight reduction of the circulation time after the detonation was observed. A mean value of  $4.3 \pm 0.10$  sec was then obtained with the limit values of 3.8 and 5.4 sec.

As the heart action in these very slightly injured animals is often quickened after the detonation this may possibly explain the reduction of the circulation time. It may be pointed out, however, that the reduction is statistically insignificant ( $0.3 > P > 0.2$ ).

In the severely injured animals the increase of the circulation time is probably dependent on the general deterioration of the circulation. It is highly probable that a slowing-up of the blood circulation in the severely damaged lungs contributes to prolong the circulation time.

*Briefly, the experiments described in this part have shown that an immediate fall in systemic blood-pressure occurs after the detonation. This fall is related to the force of the detonation and may in a few sec reach almost zero. It then rises again but is, especially in severely injured animals, lower than the initial value for some days after the detonation. A sudden circulatory collapse several hours after the detonation may be seen, but is rare. The blood-pressure drops even in animals which have been bilaterally vagotomized and sinus denervated before the experiment. In sinus denervated animals the secondary pressure increase is not seen but the blood-pressure remains low until death.*

TABLE 34. Variation of the body temperature of normal rabbits during two consecutive days.

Time for measurement	$n$	$M \pm \varepsilon(M)$	$\sigma$	
9 o'clock	first day	40	$38.9 \pm 0.05$	0.29
	second day	40	$39.0 \pm 0.04$	0.25
13 o'clock	first day	40	$39.0 \pm 0.05$	0.33
	second day	40	$39.0 \pm 0.04$	0.23
15 o'clock	first day	40	$39.0 \pm 0.04$	0.26
	second day	40	$39.1 \pm 0.04$	0.26

ture in the normal animals have been carried out during days when the temperature of the surrounding air was approximately the same as when the injured animals were examined, namely, about 18—22 °C.

The material consists of 35 animals, of which 23 had severe injuries (quotient of haemorrhage for right lung > 1.50) and 12 slight injuries (quotient of haemorrhage < 1.50).

The rectal temperature of these animals has been measured repeatedly during the days immediately before the detonation. After the detonation the temperature has again been measured as soon as possible, usually within 5 or 10 minutes. In animals on which also blood-pressure determinations have been carried out, the initial temperature value after the detonation has always been determined before the animal has been placed in the thermobox of the blood-pressure device. During the first hour after the detonation the temperature has usually been measured every 5 to 15 minute. If, after this time, it is still considerably below the initial value, it has been controlled at intervals of 15—30 minutes, until the initial value has been reached again or the value has become constant. Otherwise determinations have been carried out 2, 4, 6, 24 and 48 hours after the detonation.

Tables 35 and 36 give the mean values of the rectal temperature at different times after the detonation of slightly and severely injured animals respectively.

For the statistical analysis of the material the percental deviation of the temperature from an initial value has been calculated. The mean value of all determinations in one and the same animal before the detonation, has been chosen as initial value.

In both groups a fairly great rise in temperature is seen in several

TABLE 35. Mean value of body temperature at different times after the detonation in rabbits with slight lung injuries (quotient of haemorrhage for right lung  $<1.50$ ).

	Before detona- tion	Time after detonation						
		15 min	1 hour	2 hours	4 hours	6 hours	24 hours	48 hours
<i>n</i>	10	4	8	7	5	9	8	4
<i>M</i>	38.9	39.4	38.4	38.3	38.5	38.8	38.4	38.6
$\varepsilon(M)$	0.15	0.54	0.25	0.18	0.21	0.13	0.19	0.11
$\sigma$	0.47	1.07	0.70	0.48	0.47	0.40	0.53	0.22

animals during the 15 minutes immediately after the detonation. As regards the group of severely injured animals this increase is statistically highly probable ( $0.01 > P > 0.001$ ), but is insignificant in those slightly injured ( $0.4 > P > 0.3$ ). *After this there is generally a fall of temperature to subnormal level.* This reduction is much more prominent in the severely injured animals, where the temperature in several cases falls below  $37^{\circ}\text{C}$ . It is then subnormal in most animals during the next 24 hours.

*The difference in temperature between normal animals and those injured by blast is statistically significant both regarding the severely injured animals ( $P < 0.001$ ) and those slightly injured ( $P < 0.001$ ).* Between these two groups, however, there is no statistical difference ( $0.6 > P > 0.5$ ).

It is clear from table 35 that the temperature of the slightly injured animals is lowest about 2 hours after the detonation, when

TABLE 36. Mean value of body temperature at different times after the detonation in rabbits with severe lung injuries (quotient of haemorrhage for right lung  $>1.50$ ).

	Before detona- tion	Time after detonation								
		15 min	30 min	45 min	1 hour	2 hours	4 hours	6 hours	24 hours	48 hours
<i>n</i>	22	9	9	10	20	17	17	16	20	9
<i>M</i>	38.8	39.6	38.6	38.2	38.1	38.2	38.5	38.8	38.6	38.5
$\varepsilon(M)$	0.07	0.24	0.18	0.22	0.17	0.16	0.20	0.19	0.99	0.23
$\sigma$	0.33	0.73	0.53	0.69	0.74	0.67	0.82	0.75	0.44	0.69

there is an average reduction of  $0.6^{\circ}\text{C}$  as compared to the initial value. This difference is statistically probable ( $0.05 > P > 0.02$ ). In those severely injured the fall in temperature is greatest between 1 and 2 hours after the detonation. The reduction is statistically significant ( $P < 0.001$ ).

### Discussion.

In severely injured animals a rise in body temperature is noticed immediately after the detonation which rapidly gives way for a sub-normal temperature. A fall in temperature is often seen also in animals with only slight blast-injuries. When the body temperature is low the blood vessels of the ears are maximally contracted, and the ears are cold.

The rise in temperature following immediately after the detonation is quite difficult to explain. Possibly it may be due to psychic factors resulting from sensations caused by the detonation. On the other hand, it seems highly improbable that the heat impulse from the detonation should play an important role, as it is much too brief, and moreover, at the distance from the charge where the animals were placed, it has no great intensity. This is proved, for instance, by the fact that only a few animals have had their furs singed. Also a direct effect by the blast wave upon the temperature regulation centre is possible as the increase of pressure may cause temporary changes of temperature similar to those which are known to follow lesions to the hypothalamus region, described by a number of authors (see surveys by ISENSCHMID 1926, CLARK, MAGOUN and RANSON 1939, RANSON and MAGOUN 1939 and DUNCAN and BLALOCK 1942). In cerebral concussion an increase of the body temperature is generally found (WOODHALL 1936).

Concerning the fall in temperature occurring later, this may be an expression of the predominance of vagus after the detonation. In vagotomy the body temperature is usually low owing to a lowered basal metabolism and to circulatory changes with low blood-pressure and slow pulse. The general deterioration of the circulation in the animals injured by blast manifesting itself in the above-mentioned changes as bradycardia, reduction of blood-pressure, and prolonged circulation time would also constitute important reasons for the reduction of the body temperature. The importance of the function of the circulation organs for the heat regulation has thus been

emphasized by FREUND (1926) among others, who says that "die grösste Schädigung der wärmereregulatorischen Leistungen ist in Kreislaufschädigungen mit Blutdrucksenkung zu suchen. Wir kennen keine Art der Blutdruckssenkung — centralen oder peripheren Ursprungs — bei der nicht die Körpertemperatur abstürzt".

It may be pointed out in this connection that earlier investigations have shown that the body temperature is often lowered after severe trauma (KINNAMAN 1903, RICCA *et coll.* 1945, and others). TABOR and ROSENTHAL (1947) state, that the fall in temperature due to haemorrhage has been shown to be the result of a diminished capacity for oxygen transport.

Several authors (see for instance BINTAKYS 1947) have stressed the importance for the thermo-regulation of emitting heat through the respiratory passages. An increase of the respiration is accompanied by an increased emission of heat. Owing to the poor ventilation in animals with severe lung blast lesions, however, the increased respiratory rate in these animals does not seem to have any decisive importance to the heat regulation.

## CHAPTER 16.

### Changes in Blood Chemistry in Blast Injury with Special Regard to Possible Traumatic Shock.

Published data concerning morphological and chemical blood changes in blast injury are exceedingly sparse. This applies to clinical as well as experimental investigations.

HADFIELD and CHRISTIE (1941) describe a fatal case of lung blast where the alkali reserve was 60.5 vol% and blood urea 86 mg% 42 hours after exposure to bomb blast, and 9 hours before death.

ZAKHAROV (1943) has studied the changes in some different components of the blood and spinal fluid in air concussion. The potassium content was lowered while the calcium content for the main part remained unchanged, thus reducing the K/Ca-quotient. Glucose, chlorides, non protein nitrogen and cholesterol remained mainly at normal values.

GAGE (1945), finally, mentions some laboratory findings in 19 cases of severe immersion blast. The haemoglobin content and number of red blood cells were normal in most of the cases. Determinations of haematocrite and plasma volume showed normal values except in two cases.



HOOKE (1924) has determined the alkali reserve in some of the dogs used in his experiments. In one of the animals with primary shock the alkali reserve was normal 5 minutes after the explosion (69.1 vol %), while  $1\frac{3}{4}$  hours after the explosion it had dropped to 43.8 vol %. One dog, not shocked, had an increased alkali reserve. Another dog, on the contrary, also not shocked, showed a marked decrease 9 minutes after the detonation. HOOKE points out that a lowered alkali reserve cannot be regarded as an essential feature of air concussion shock. He refers also to PENFIELD (1919) who states that the alkali reserve decreases rapidly if a low arterial pressure is maintained through haemorrhage.

### **Blast shock.**

The question whether traumatic shock occurs in blast injury is not yet proved. Most authors who have examined people injured by blast have indeed stated that these were suffering from different degrees of shock. Whether this has been a direct result of the actual blast injury, however, has been difficult to decide as the injuries have not generally been pure blast injuries but combined with wounds, burns etc. BLALOCK and DUNCAN (1942) in a survey of different types of traumatic shock have also briefly touched upon the subject of blast injuries but do not contribute to solve the problem. TUNBRIDGE (1945) maintains, that "contrary to expectation, circulatory shock is not a feature of blast injury".

It seems of interest, therefore, to find out whether the blood changes generally occurring in shock could be observed in animals injured by blast. To begin with a short survey will be given of earlier studies on blood chemistry in traumatic shock.

#### *Survey of changes in blood chemistry in traumatic shock.*

Owing to the great number of papers dealing with traumatic shock, this review does not at all pretend to be complete.<sup>1</sup>

It was generally thought earlier that an increase of the haemoglobin content, a haemoconcentration, was one of the principal symptoms of traumatic shock (cf. MOON 1938). This would not be the case, however, and it is nowadays fairly generally believed that haemoconcentration mainly exists only in burn shock and crush injury (cf. CROOKE and MORRIS 1944).

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<sup>1</sup> Only during the last world war more than 2000 papers concerning traumatic shock have been published of which about 800 are experimental investigations.

The changes of plasma *chlorides* in shock and in haemorrhage without shock have been studied by a number of authors. The results do not entirely agree, but it has generally been found that the chloride content is decreased in shock (BISGARD *et coll.* 1938, MOON *et coll.* 1941, COLE *et coll.* 1944) while, on the other hand, it is slightly increased in haemorrhage (BLACK 1940, MOON *et coll.* 1941, PRICE *et coll.* 1941). GREGERSEN (1946) states, finally, that the plasma chloride content in dogs does not change in shock caused by trauma or haemorrhage.

The content of *total proteins in plasma*, during the first stages of shock at least, seems little changed (BEARD and BLALOCK 1931, CREMER 1942, KØSTER 1943). If shock is combined with dehydration a concentration of plasma takes place with a relative increase of the protein content (DAVIS 1940, KØSTER 1943). In greater haemorrhages the organism may lose a considerable amount of protein, and the protein content in the plasma then decreases (KØSTER 1943).

A reduction of pH of blood and a low *alkali reserve* are considered usual in traumatic shock (GUTMAN *et coll.* 1941, CURNAND *et coll.* 1943, COLE *et coll.* 1943, GREGERSEN 1946, ROOT *et coll.* 1947, and others). This is supposed to be due primarily to an increase of the lactic acid content as a result of a deteriorated carbohydrate metabolism caused by a relative tissue anoxia with acidosis as a consequence (MEYLER, 1939, GUTMAN *et coll.* 1941, ENGEL *et coll.* 1943, COLE *et coll.* 1944, BEATTY 1945). Also in haemorrhage without shock a reduced alkali reserve seems to be a common occurrence. PENFIELD (1919) believes, as mentioned above, that if a low arterial blood-pressure is caused by haemorrhage the alkali reserve decreases immediately, and PRICE *et coll.* (1941) point out that also in haemorrhage there will be insufficient oxidation particularly in the muscles which would result in an increase of the lactic acid followed by a reduction of the alkali reserve. GOVIER and GREER (1941) have shown a significant increase of ketone acids in the blood of dogs shocked by blood letting.

*Blood sugar* seems generally raised in earlier stages of traumatic shock but decreases later especially in the terminal stadium (ENGEL *et coll.* 1943, HAIST and HAMILTON 1944, and others).

*Non protein nitrogen.* Decreased blood flow through the kidneys caused by vasoconstriction is considered by LAUSON, BRADLEY and CURNAND (1944) to be an important compensatory mechanism in maintaining a normal blood-pressure in shock. As a result of the reduced blood flow through the kidneys oliguria or even anuria follows, causing an increase of non protein nitrogen (MOON *et coll.* 1941, EBERT *et coll.* 1942, GREGERSEN 1946, and others).

An increase of the *phosphate* content of plasma, which is raised even after extensive haemorrhages, has been described by MYLON *et coll.* 1943, DUNCAN 1943, COLE *et coll.* 1944, Mc SHAN *et coll.* 1945, MEYER *et coll.* 1946, GREGERSEN 1946, ALLISON *et coll.* 1947, NASTUK 1947 and SELIGMAN *et coll.* 1947. The phosphate content of plasma, however, is increased even in anaesthesia (LIPOW, WEAVER and READ 1929, MACKAY 1929/30).

Changes in the *potassium content of plasma*, finally, have been considered of great importance in shock, and potassium poisoning has actually been regarded as a direct cause of death (cf. *e. g.* TABOR and ROSENTHAL 1945). A number of authors have found an increase of potassium in plasma (SCUDDER 1940, CLARKE

and CLEGHORN 1942, CREMER 1942, and others), but generally the investigations have shown that increase of potassium in plasma is a terminal occurrence which only appears immediately before death possibly in connection with premortal changes in the tissues (ZWEMER and SCUDDER 1938, MANERY and SOLANDT 1943, HOLMES 1947, and others).

### Own investigations.

Of technical reasons all the components mentioned above have not been analyzed in each animal but the material has been divided into two groups.

*Group I:* This group consists of 23 animals in all, namely, 15 with severe injuries (quotients of haemorrhage for right lung  $> 1.50$ ) and 8 with slight injuries (quotients of haemorrhage  $< 1.50$ ). In blood from these animals haemoglobin has been determined together with chlorides, alkali reserve and total protein. In addition to this the cell volume of blood and blood sugar have been determined in some of the animals.

Blood samples have been taken 24 hours before the detonation and also 2 and 6 and 24 hours after it. In some cases samples have been taken also after 48 hours and after 6 days. All animals within this group, both experimental and control animals, have been unanaesthetized.

The blood volume of the rabbit, according to AGNER and VON PORAT (1947) is 63—73 ml per kg body weight. SCHULTZE (1925), in a review of the literature on this subject gives the limit values 43.5 and 81 g of blood per kg body weight.

In rabbits with body weights of between 2 and 3 kg the total blood content, if using the mean of the values given by AGNER and VON PORAT, is thus only about 140—200 ml. It is obvious, therefore, that taking a number of blood samples each of about 5 or 7 ml of blood may cause changes in blood chemistry similar to those usually seen after a haemorrhage.

The changes which might have been caused merely by blood letting have been studied in a number of normal animals. Blood samples have been taken at the same times as in the blasted animals. In order to compare the two groups the amount of blood from either group must be as near as possible the same and must be taken at one and the same time. The mean values for the blood quantities taken at different times are shown in table 37.

TABLE 37. Quantity of blood (in ml) taken at various times from the control rabbits and the rabbits injured by blast.

Time for bleeding in relation to detonation	Control rabbits			Rabbits injured by blast		
	<i>n</i>	$M \pm \varepsilon(M)$	$\sigma$	<i>n</i>	$M \pm \varepsilon(M)$	$\sigma$
24 hours before	13	$7.4 \pm 0.43$ ml	1.56	21	$6.6 \pm 0.49$ ml	2.23
2 hours after	14	$8.5 \pm 0.43$ "	1.62	17	$5.4 \pm 0.60$ "	2.47
6 " "	13	$6.7 \pm 0.39$ "	1.41	20	$4.6 \pm 0.39$ "	1.74
24 " "	12	$7.4 \pm 0.44$ "	1.54	20	$5.1 \pm 0.37$ "	1.66
48 " "	14	$7.5 \pm 0.45$ "	1.70	15	$5.3 \pm 0.32$ "	1.28

*Group II:* This group consists of 18 animals in which non protein nitrogen, and the content in plasma of creatinine, phosphorus and potassium have been determined. Blood has been taken only once from each animal, usually when the animal was killed. Three of the animals within this group have died from their injuries. Of the others, 8 had severe lesions and 7 slight lesions. Nineteen normal rabbits have been used as controls. All animals within this group have been anaesthetized with urethane (1.4—1.6 g per kg body weight).

### Blood chemistry in blasted animals.

#### Haemoglobin.

The haemoglobin content is subject to quite great variations even in normal rabbits. Because of this the values found by different authors vary quite considerably. This may be due to the fact that different methods with different standards have been used. But even the values obtained by one and the same author vary quite considerably. A survey of limit values for haemoglobin in the rabbit is given by JAFFÉ (1931).

The present author has determined the haemoglobin content in 65 normal rabbits of male sex whereby a mean value of  $13.79 \pm 0.19$  g Hb/100 ml ( $\sigma = 1.50$ ) was obtained.

In animals injured by blast as in the control animals there is a progressive reduction of the haemoglobin content, as shown in table 38. This is due to the blood loss caused by blood letting and to the slow formation of new haemoglobin (cf. SCHEFFER and BÖHM 1933). The table gives the mean value of the percental deviation of haemoglobin from the initial value *i. e.* the haemoglobin content of the blood taken 24 hours before the detonation.

TABLE 38. *Haemoglobin content in blood* of control rabbits and rabbits injured by blast. The values are given in per cent of the initial value obtained 24 hours before detonation.

Initial value	Control rabbits			Rabbits with severe blast injuries		
	<i>n</i>	$M \pm \varepsilon (M)$	$\sigma$	<i>n</i>	$M \pm \varepsilon (M)$	$\sigma$
	14	$14.09 \pm 0.38$ g/100 ml	1.43	17	$13.41 \pm 0.43$ g/100 ml	1.77
Time after detonation:						
2 hours .....	14	$-10.3 \pm 1.1 \%$	4.1	14	$-15.9 \pm 2.7 \%$	10.2
6 " .....	13	$-15.5 \pm 1.3 \%$	4.8	15	$-23.2 \pm 2.8 \%$	10.7
24 " .....	12	$-19.6 \pm 2.6 \%$	9.1	13	$-27.6 \pm 3.6 \%$	12.9
48 " .....	14	$-24.0 \pm 1.8 \%$	6.7	9	$-17.4 \pm 3.5 \%$	10.5

As shown in the table the reduction of the haemoglobin content is greatest in the severely injured animals. The difference between these and the control animals is statistically significant ( $P < 0.001$ ).

#### Cell volume of blood.

The cell volume of blood has been determined in a few cases with slight and severe blast injury.

The reduction of the haematocrite value runs fairly parallel with the reduction of the haemoglobin concentration.

#### Plasma chlorides.

A determination of the chloride content and its normal variations in 38 rabbits gave a mean value of  $102.0 \pm 0.8$  m mol ( $\sigma = 4.9$ ). It may be mentioned that COLE *et coll.* (1941) found values between 84 and 110 (mean value 104) m mol in 30 normal non-fasting animals, and in 11 animals which had fasted 24 hours before being bled they found between 92 and 112 (mean value 105) m mol.

The mean values for the percental deviations of the plasma chloride values from the initial value in the control animals and in the blasted animals are found in table 39.

It is seen from the table that the plasma chloride content increases both in the normal animals and in animals injured by blast. The dispersion, however, is considerable, and in some animals there is instead a slight decrease of the chloride content.

TABLE 39. *Chloride content in plasma* of control rabbits and rabbits injured by blast. The values are given in per cent of the initial value obtained 24 hours before detonation.

	Control rabbits			Rabbits with slight blast injuries			Rabbits with severe blast injuries		
	<i>n</i>	$M \pm \varepsilon(M)$	$\sigma$	<i>n</i>	$M \pm \varepsilon(M)$	$\sigma$	<i>n</i>	$M \pm \varepsilon(M)$	$\sigma$
	13	$103.7 \pm 1.4$ m mol	5.1	6	$106.0 \pm 1.3$ m mol	3.07	15	$106.0 \pm 2.3$ m mol	8.98
Initial value									
Time after detonation:									
2 hours ...	13	$-2.0 \pm 0.8 \%$	2.9	3	$+2.6 \pm 2.8 \%$	4.8	11	$+1.2 \pm 4.1 \%$	13.5
6 " ...	13	$+3.2 \pm 0.9 \%$	3.2	6	$+4.2 \pm 1.6 \%$	3.9	14	$+4.7 \pm 2.3 \%$	8.7
24 " ...	13	$+0.9 \pm 0.9 \%$	3.3	6	$+4.3 \pm 1.6 \%$	3.9	12	$+5.4 \pm 2.4 \%$	8.1
48 " ...	11	$+0.3 \pm 1.3 \%$	4.2	6	$+0.6 \pm 1.9 \%$	4.6	9	$+0.9 \pm 1.1 \%$	3.2

The difference between normal and blasted animals is insignificant ( $0.2 > P > 0.05$ ). The same applies to the difference between slightly and severely injured animals ( $0.2 > P > 0.05$ ).

#### Total protein in plasma.

In 55 normal non-fasting animals the protein content in plasma reached an average of  $6.59 \pm 0.11$  g/100 ml ( $\sigma = 0.80$ ), which value corresponds quite well with the mean value given by COLE *et coll.* (1944) of 6.18 g/100 ml (limit values 5.4—7.0 g/100 ml) in non-fasting

TABLE 40. *Total protein content in plasma* of control rabbits and rabbits injured by blast. The values are given in per cent of the initial value obtained 24 hours before detonation.

	Control rabbits			Rabbits with slight blast injuries			Rabbits with severe blast injuries		
	<i>n</i>	$M \pm \varepsilon(M)$	$\sigma$	<i>n</i>	$M \pm \varepsilon(M)$	$\sigma$	<i>n</i>	$M \pm \varepsilon(M)$	$\sigma$
	12	$6.91 \pm 0.05$ g/100 ml	0.18	7	$6.82 \pm 0.2$ g/100 ml	0.49	15	$5.88 \pm 0.2$ g/100 ml	0.66
Initial value									
Time after detonation:									
2 hours ...	12	$-6.7 \pm 1.5 \%$	5.1	4	$-15.8 \pm 2.3 \%$	4.6	12	$-9.9 \pm 2.8 \%$	9.7
6 " ...	11	$-12.3 \pm 2.2 \%$	7.3	6	$-13.7 \pm 1.6 \%$	3.8	15	$-12.8 \pm 2.5 \%$	9.5
24 " ...	9	$-10.0 \pm 1.7 \%$	5.2	7	$-11.2 \pm 1.9 \%$	5.1	13	$-3.1 \pm 1.3 \%$	4.8
48 " ...	12	$-8.1 \pm 2.0 \%$	7.0	5	$-11.1 \pm 2.8 \%$	6.4	10	$-3.7 \pm 3.6 \%$	11.3

TABLE 41. *Alkali reserve* of control rabbits and rabbits injured by blast. The values are given in per cent of the initial value obtained 24 hours before detonation.

	Control rabbits			Rabbits with slight blast injuries			Rabbits with severe blast injuries		
	<i>n</i>	$M \pm \varepsilon(M)$	$\sigma$	<i>n</i>	$M \pm \varepsilon(M)$	$\sigma$	<i>n</i>	$M \pm \varepsilon(M)$	$\sigma$
	12	$53.7 \pm 1.7$ vol %	5.8	7	$42.0 \pm 3.7$ vol %	9.9	14	$41.0 \pm 2.7$ vol %	10.1
Initial value									
Time after detonation:									
2 hours ...	12	$+10.2 \pm 2.8 \%$	9.6	4	$-15.5 \pm 4.9 \%$	9.7	11	$-9.9 \pm 9.3 \%$	30.7
6 " ...	10	$+4.5 \pm 2.7 \%$	8.6	7	$+11.2 \pm 11.2 \%$	29.5	13	$-3.7 \pm 5.9 \%$	21.4
24 " ...	10	$+9.4 \pm 3.7 \%$	11.8	7	$+19.1 \pm 5.8 \%$	15.3	11	$-15.4 \pm 7.2 \%$	23.8
48 " ...	12	$+10.9 \pm 6.2 \%$	21.4	6	$+17.7 \pm 7.8 \%$	19.0	8	$-12.6 \pm 6.7 \%$	19.0

animals and of 6.37 g/100 ml (limit values 5.8—7.2 g/100 ml) in fasting animals.

The percental deviations of the protein content from the initial value in blasted animals and controls are shown in table 40.

In the control animals as well as in the blasted animals there is thus a reduction of the protein content of the plasma, which is most marked at 2 and 6 hours after the detonation. No statistical difference exists, however, between these two values ( $0.2 > P > 0.05$ ). As far as the severely injured animals only are concerned, and these are compared with the control animals,  $P > 0.2$  is obtained. The reduction of plasma protein, therefore, must be attributed chiefly to the blood loss caused by the blood letting.

### Alkali reserve.

HOOKE's investigations (1924) give no certain criterion for the assumption that a decreased alkali reserve should be of major importance in air concussion shock.

He even found in a case of blast injury with no shock a noticeable increase of the carbon dioxide binding capacity.

The alkali reserve varies within quite broad limits even in normal rabbits, and much more than in man (cf. MARKEES and MENCZER 1946). In 50 normal non-fasting animals the present author obtained the limit values of 29.5 and 70.5 vol % (mean value  $47.3 \pm 1.50$  vol %,  $\sigma = 10.6$ ). As a comparison may be mentioned that the values in MARKEES and MENCZER's material were between

21.7 and 54.88 vol %, and that ZUNZ and CRACIUNESCU (1938) give 39.5—57.5 vol % as normal values for rabbits.

In the control animals, which have been bled at the same time as the experimental animals, there is a moderate increase of the alkali reserve in most cases. In the slightly injured rabbits there will be a moderate reduction to begin with, but already 6 hours after the detonation most of the animals show an increase up to and over the initial value. This increase remains for the next 48 hours. *In the severely injured animals, finally, a reduction of the alkali reserve is found in most of the animals already 2 hours after the detonation.* The increase will be progressive in the majority of cases.

For statistical study of the material the percental deviation from the initial value before the detonation has been determined for each animal. The means of these percental values obtained at different times after the detonation are found in table 41.

Variance analysis including the controls and the slightly injured animals shows that a significant difference exists between these groups ( $P < 0.001$ ). The decrease of the alkali reserve in severely injured animals is also statistically significant ( $P < 0.001$ ). In the slightly injured animals the initial reduction is statistically probable ( $P = 0.05$ ). The difference between this value (2 hours after the detonation) and the next values is highly probable ( $0.01 > P > 0.001$ ). No statistical difference exists between the mean differences obtained at different times in the control animals ( $P > 0.2$ ). The same applies to the group of severely injured animals ( $P > 0.2$ ).

### Blood sugar.

Blood sugar has been determined in some of the animals belonging to group I. The results from these investigations and also from determinations of blood sugar on some ten other rabbits injured by blast show that a moderate increase generally exists. The increase, however, is not seen throughout the whole material, and in some cases even a slight reduction has been noticed probably to some extent due to the fact that the severely injured animals do not feed during the first 12 to 24 hours.

### Non protein nitrogen.

In the 18 animals injured by blast and belonging to group II (see page 175) non protein nitrogen was determined when the animals



were killed. A mean value of  $49.3 \pm 7.0$  mg/100 ml was obtained. No significant difference existed between animals with slight injuries ( $M = 51.0 \pm 3.3$  mg/100 ml) and those with severe injuries ( $M = 48.3 \pm 3.5$  mg/100 ml), ( $0.6 > P > 0.5$ ). In 19 control animals a mean value of  $40.5 \pm 1.4$  mg/100 ml was obtained. The values are somewhat higher than in unanaesthetized animals owing, among other things, to the fact that the anaesthetic contains about 16 per cent nitrogen.

The difference between the animals injured by blast and the normal animals is insignificant ( $0.3 > P > 0.2$ ).

For the three animals, finally, which have died from their injuries the mean value of  $54.3 \pm 6.8$  mg/100 ml was obtained. The difference between these animals and the normal animals is probable ( $P = 0.05$ ).

#### Plasma creatinine.

In 17 animals injured by blast the mean value for plasma creatinine was  $1.62 \pm 0.11$  mg/100 ml. No difference existed between the two groups of slightly and severely injured animals.

In the 19 control animals the mean value of  $1.52 \pm 0.09$  mg/100 ml was obtained. The difference between blasted and normal animals is statistically insignificant ( $0.5 > P > 0.4$ ).

In the three mortally injured animals the mean value of  $1.83 \pm 0.42$  mg/100 ml was obtained. The increase is insignificant ( $0.5 > P > 0.4$ ).

#### Plasma phosphorus.

Determinations of the phosphate content in plasma in 19 normal rabbits gave a mean value of  $4.69 \pm 0.29$  mg/100 ml.

In the 18 animals injured by blast a mean value of  $5.39 \pm 0.79$  mg/100 ml was obtained. The mean value for 7 slightly injured animals was  $3.00 \pm 0.58$  mg/100 ml and for 7 animals with severe, but not fatal, injuries it was  $5.58 \pm 0.53$  mg/100 ml.

The decrease of the phosphorus content in the slightly injured animals is statistically probable ( $0.02 > P > 0.01$ ). The reason for this reduction is not quite clear. CAMPBELL, BROWN and GOLLAN (1948), however, have shown that the content of inorganic phosphorus in plasma decreases in hyperventilation. It seems probable, therefore,

that the considerable acceleration of respiration in these slightly injured animals may be of importance here.

The difference in the phosphate content between normal animals and severely, but not fatally, injured animals is statistically insignificant ( $0.2 > P > 0.1$ ). In animals with fatal injuries, on the other hand, there is an increase of the phosphate content which is statistically highly probable ( $0.01 > P > 0.001$ ).

### Potassium in plasma.

In the rabbits injured by blast an increase of the potassium content in plasma has been observed. The mean value for the 17 animals is  $7.41 \pm 0.8$  mol. The mean value for the 18 control animals is  $5.05 \pm 0.27$  mol.

The increase is mainly due to the three animals which died from the blast injuries and which show a very sharp increase of plasma potassium.

The slightly injured and those with severe, though not fatal, lung injuries, show only a slight increase. The mean values for the two groups are  $5.99 \pm 0.64$  and  $6.19 \pm 0.95$  m mol respectively. The increase is statistically insignificant ( $0.2 > P > 0.1$ ) in both groups.

The mean value of the plasma potassium content in the mortally injured animals is  $13.07 \pm 1.13$  m mol. This increase compared to the normal animals is statistically significant ( $P < 0.001$ ).

### Discussion.

The present investigation has thus shown that certain changes in blood chemistry occur in blast injury.

The reduction of the haemoglobin content which has been observed in all the animals depends, first and foremost, on the repeated blood letting. The reduction is greatest in the severely injured animals, which have also "lost" a rather considerable amount of blood in the lungs, and, though in a much less degree, in the gastro-intestinal canal.

The average body weight of the severely injured animals is 2 190 g. If the blood content of rabbits, in accordance with the above mentioned, is roughly estimated at 7 per cent of the body weight the mean weight of the blood in these animals will be about 150 g. Twenty-four hours after the detonation the average reduction of the haemoglobin

content is 8.0 per cent greater in the severely injured animals than in the control animals. Supposing the reduction of the haemoglobin content being dependent on the blood loss only, this would then be about 8 per cent greater in the severely injured animals, *i. e.* corresponding to an average of about 12 g of blood. The mean value of the weight increase of left and right lung, obtained by weighing the lungs, is 8.3 g for those animals in this group which were killed 24 hours after the detonation. Considering the fairly great errors in these determinations the values correspond fairly well and speak against the existence of, at any rate, a more prominent haemoconcentration (cf. also page 64).

The difference in the decrease of haemoglobin between the control animals and the animals injured by blast increases as time goes on (—5.6, —7.7 and —8.3 per cent at 2, 6 and 24 hours after the detonation respectively). This increase indicates a continued bleeding in the lungs, a fact which also has been proved through decreasing haemoglobin values in a number of cases where only a few drops of blood have been taken each time for the determination. The progressive bleeding in the lungs has also been pointed out by ROBERTS (1940), HADFIELD (1941), and HADFIELD and CHRISTIE (1941). On post mortem examination it has actually been observed that the great original haemorrhagic regions are surrounded by smaller fresh haemorrhages in the area bordering on to uninjured tissues.

A certain increase of the chloride content in plasma is seen as well as a reduction of the total protein content. These changes, however, are observed both in the control animals and in the blasted animals.

Therefore the reduction of haemoglobin, cell volume and total protein in plasma as well as the increase of chloride would, mainly, be due to the blood loss.

The increase of blood sugar usually seen in the blasted animals may be due to an impaired oxidation of glucose in the tissues as a result of the general anoxia. It may, however, also be a sign of an increased secretion of adrenaline.

The alkali reserve decreases fairly regularly in severely injured animals. The reason for this would, first and foremost, be a reduced elimination of carbon dioxide through the lungs owing partly to the general circulatory changes, partly and perhaps to an even greater extent, to the insufficient gas exchange in the severely injured lungs. In consequence of this there will be a tissue anoxia and an in-

Investigations by the present author into the effects of the blast wave upon respiration and heart action have clearly shown that such radical changes occur that these must be considered the immediate cause of death. Before further discussing these points of view, however, some factors must first be mentioned which in the literature were regarded as possible causes of death.

### 1. Carbon monoxide poisoning.

As mentioned already in the first chapter the explosion gases, particularly the carbon monoxide, were at first considered important factors in causing injury in explosions. A great number of authors have also been of the opinion that death in explosion disasters was caused by carbon monoxide poisoning. In recent times this opinion has been supported by *e. g.* LOGAN (1939) and HADFIELD *et coll.* (1940), who in a number of cases of blast injury found a high carbon monoxide saturation in the blood.

The existence of carbon monoxide poisoning after detonations has been denied, however, by several authors for instance BAHIER (1905), RAVAUT (1915), WILSON and TUNBRIDGE (1943) and others. It certainly seems improbable that the carbon monoxide in the explosion gases, except in special circumstances, for instance in confined spaces, could play an important role in causing injury.

In order to exclude carbon monoxide poisoning as a contributing factor in causing the changes described in this paper, the carbon monoxide content in blood has been determined in a number of animals exposed in the field or in the detonation chamber. Blood has been taken from the animals 15 minutes after the detonation. The animals had been left for 8 or 10 minutes after the explosion at the place of the explosion or in the cage on the pendulum of the detonation chamber. It should be noted that in the experiments in the detonation chamber the animal cage follows the pendulum when this swings out. The risk of carbon monoxide poisoning is of course greater inside the detonation chamber than outside its opening.

In 8 animals exposed in the field to blast waves from 1 kg charges, all obtaining severe lung lesions (three of these animals died from the injuries) a carbon monoxide content was determined which varied in the different animals between 0 and 0.32 vol % the mean being  $0.10 \pm 0.03$  vol %.

The carbon monoxide content in the blood has also been determined in 17 animals of which almost all had obtained severe lung lesions after exposure to blast in the detonation chamber. In two of these animals only can the lesions be characterized as moderate. The carbon monoxide saturation varied between 0 and 0.8 vol % with a mean value of  $0.22 \pm 0.05$  vol %.

As it would be necessary to have a carbon monoxide saturation in the blood of at least 30 per cent (*i. e.*  $> 4.5$  vol % of CO) in order to cause serious acute symptoms, it is obvious that no possibility whatever of fatal carbon monoxide poisoning has existed in the present material.

## 2. Air embolism.

REYNAUD (1887), GAUDIN (1887), SCHER (1941), and others, believe that during the high-pressure phase of blast an excess amount of gas would be dissolved in the blood. The gas would again be released during the following low-pressure phase, thus causing air emboli. Air emboli, however, have never been observed in post mortem examinations. This has been explained by SCHER as probably due to the fact that when the atmospheric pressure has again become stabilized after the detonation it would act like a recompression in comparison to the preceding suction wave and the gas would rapidly be dissolved again.

No signs of air emboli have been found in the present material. It seems highly improbable therefore, that air emboli of any significance could be set free during this very short low-pressure phase. Even if this were possible, however, the emboli would never have time to cause serious changes if they in accordance with SCHER's opinion were dissolved in the blood again immediately after the detonation.

On the other hand, it does not seem entirely improbable that small quantities of air could be forced in through the injured lung capillaries under the influence of an increased intrapulmonary pressure (see *e.g.* POLAK and ADAMS 1932) — cf. also CARLTON, RASMUSSEN and ADAMS (1945), who were able to produce coronary air embolism as a result of a moderate pressure rise in the lungs caused by blowing air into trachea. There would be no time to force in a sufficient quantity of air, however, and air emboli as a cause of death in blast injury can with all probability be ruled out.

### 3. Fat embolism.

McKIBBEN (1915), who has examined the brains and other organs from a number of dogs used in Hooker's blast experiments, for the existence of intravascular fat, found small fat droplets in the blood vessels, particularly in the brain and lungs, but also in other organs. He points out, however, that such fat could be found also in normal animals. COHEN and BISKIND (1946) found intravascular fat in the lungs in 8 out of 11 persons killed by blast injuries, but are of opinion that this is of no significance to the lung injury or to the clinical picture of blast injury. Also ROBB-SMITH (1941) has found pulmonary fat emboli in blast and believes that the symptoms in blast injury may be due to a combination of pulmonary concussion and fat embolism.

The present animal material has been examined with regard to the existence of fat embolism. The lungs from a number of severely injured animals have been sectioned and stained with Sudan III.

Intravascular fat has been observed in some of the animals. In all the cases there has only been smaller quantities of fat in the thinner blood vessels of the lung. In one and the same slide only a few vessels have been filled with fat while other vessels within the same section have been quite free from fat. No significant difference has been found in the quantity of intravascular fat found in animals which have died from the injuries and in less severely injured animals.

In sections of lungs from a number of normal control animals no intravascular fat has been observed.

The examination has thus shown that fat emboli may be found in blast injury but that the quantity of intravascular fat is so small that it must be regarded as without importance as a cause of death.

### 4. Respiratory and circulatory failure.

The imperfect knowledge of the changes in respiration and circulation caused by the blast wave, would most likely be the reason why the importance of respiratory and circulatory failure in causing death has hardly been considered previously. Some authors have been inclined to believe that death was caused by the blast wave acting upon vital centra (MOTT 1916, HADFIELD *et coll.* 1940, STEWART, RUSSEL and CONE 1941, HAMLIN 1943, and others). This

view is also to some extent supported by WILSON and TUNBRIDGE (1943) who, like HADFIELD *et coll.* (1940) believe that the lung injuries are not always sufficient to cause death, but that "it seems more likely that blast produces death by interfering with some vital tissue or centre in which, from the extreme rapidity of action, structural changes are unlikely to be found".

*The investigations by the present author have shown without doubt, however, that respiratory and circulatory changes are the most important causes of death, and that the effect is chiefly elicited peripherally through the lung lesions. This opinion is supported by the observations that in many animals with maximally forced respiratory movements there has nevertheless been no ventilation worth mentioning owing to the respiratory tractus being filled with blood. These animals have died from suffocation, the central regulation mechanism being intact.*

In animals which after the detonation have made only a few respiratory efforts, suffocation is thus the direct cause of death. Investigations into the effect of blast waves upon the circulation show clearly, however, that also this is often serious enough to produce death. It is quite clear that in cases where the myocardiac lesions are severe enough to produce the above-mentioned monophasic E. C. G. curves the only result would be death. On the other hand, the experiments have shown without doubt that the animals never die from an instantaneous vagal cardiac arrest.

To decide in each particular case whether the respiratory or circulatory changes play the main part would be difficult, and seems of slight importance.

*Summarizingly it may be said that in cases where the animals have died immediately after the detonation death has been caused by a combined respiratory and circulatory failure.*

## 5. Traumatic shock.

In cases where death has followed later, for instance, hours or days after the injury, the fatal outcome has usually depended on circulatory insufficiency. *The existence of a fatal traumatic shock with its characteristic haemodynamic and chemical changes in the blood of animals which have survived the initial respiratory and circulatory changes, would probably be rare in blast injury.*

## Summary.

*Introduction* (page 7).

A survey is given of the concept of blast injury.

The purpose of the present investigation was to study the effects of shock waves from detonating high explosives on physiological functions of the organism and the relation between the physical qualities of the shock wave and the lesions caused.

*The Detonation and the Physics of the Blast Wave* (Chapter 1, page 10).

The physical properties of the blast wave are described.

*Earlier Animal Investigations* (Chapter 2, page 16).

A review is made of earlier animal experiments on blast injury and on physiological effects of blast waves.

*Theories concerning the Mechanism of Blast Injuries* (Chapter 3, page 21).

Some theories are mentioned concerning the mechanism of blast injuries and the importance of the gas content of viscera for the causation of the lesions.

*Experimental Investigations. Common Experimental Arrangements* (Chapter 4, page 23).

The blasting experiments have been performed in the field and in a specially constructed detonation chamber. In field blastings cylindrical charges of cast TNT (charge weight 0.2—200 kg) were used and in the detonation chamber charges of 2—6 g of a plastic high explosive. Rabbits were used as experimental animals. Most of them were anaesthetized with urethane.

*Methods of Analysis* (Chapter 5, page 26).

All methods used are described. The methods for recording the shock wave and for measuring the maximum pressure and impulse have not been published earlier. The methods employed for recording respiration and arterial blood-pressure have been developed for the quite special conditions of blasting experiments.



*Detonation Chamber with Impulse Pendulum* (Chapter 6, page 41).

A detonation chamber has been constructed in which it is possible to get high max. pressures and great impulses variable at will with very small charges. A detailed description is given of the chamber.

*The Errors of the Pressure and Impulse Determinations* (Chapter 7, page 46.)

The errors of the max. pressure and impulse values are discussed. They are shown to depend to a minor degree on the errors of the measuring apparatus and to the greatest part on the deviation of the pressure and impulse field from spherical symmetry (the azimuthal dispersion of the values).

*Objective Determination of the Lung Blast Injury* (Chapter 8, page 52).

The lung injuries (haemorrhages and emphysema), being the most important effects of blast, have been determined objectively in all animals used. The haemorrhages are indicated by a quotient, showing the increase of weight of the lung in relation to its initial weight, the latter having been calculated from the body weight and the lung weight in per cent of body weight. The emphysematic volume increase of the lung has been calculated in a similar way.

*Development and Localization of Blast Injury* (Chapter 9, page 56).

The general condition of the animals after the detonation and the localization of the lesions are described. Blast effects principally on internal organs; external lesions being rare in pure blast injury. Injuries to the ear drum are common and retrobulbar haemorrhages are seen in severely injured animals. Of internal injuries those to the lungs are dominating, but abdominal lesions, particularly intestinal haemorrhages, are found to be much more common than may be judged from the literature. Some characteristic localizations of the pulmonary haemorrhages are mentioned. An acute traumatic pulmonary emphysema is common but pulmonary oedema seems not to be of any greater importance.

In severely injured animals lacerations and haemorrhages in the myocardium are common.

*The Occurrence of "Rib Markings" in Lung Blast Injury* (Chapter 10, page 69).

An arrangement of the lung haemorrhages along the lines of the ribs ("rib markings") is common. In animals exposed side-on to the charge the "rib markings" are localized mainly in the lung opposite to that facing the charge and they are found to correspond to the intercostal spaces and not to the ribs. An explanation is suggested.

*Relation between the Physical Qualities of the Blast Wave and the Extent of the Blast Injury* (Chapter 11, page 75).

The relation between the extent of the pulmonary and abdominal injuries and the magnitude of the maximum pressures and impulses which acted on the animals has been studied and a fairly good correlation is shown to exist. A formula for the minimum survival distance from the charge has been calculated. Pressure-distance and impulse-distance diagrams obtained with a number of charges of different weight, are given.

*The Effects of Blast Waves on Respiration* (Chapter 12, page 95).

Respiration is shown to be highly influenced by blast waves. The respiration rate is increased and the amplitude and total ventilation diminished. Immediately after the detonation respiration is often extremely irregular. Severely injured animals often die without having drawn a single breath after the detonation.

In animals bilaterally vagotomized before the detonation there is little or no increase of the respiration rate but the amplitude is increased. No obvious respiratory changes are seen in animals which have been protected, except for their heads, against the shock wave.

*The Effects of Blast Waves on Heart Action* (Chapter 13, page 119).

In spite of the heart action being less sensitive to shock waves than the respiration, the changes in the electrocardiogram may, nevertheless, be considerable in severely injured animals. An instantaneous bradycardia is the most prominent feature. It is shown to depend principally on vagal reflexes probably elicited in the severely injured and distended lungs. The heart rate, however, always increases again after the initial bradycardia and an immediate cardiac standstill never occurred. After violent detonations changes are seen indicating severe myocardiac lesion.

*Effects on the Peripheral Circulation* (Chapter 14, page 149).

An almost instantaneous fall in blood-pressure occurs after the detonation and it is dependent on the violence of the detonation. A similar fall in blood-pressure is seen in bilaterally vagotomized and sinus denervated animals. After the initial fall the pressure in surviving animals increases again but may still for a day or two be lower than the initial value. In sinus denervated animals the pressure remains low, until the animal dies. The fall in systemic blood-pressure is supposed to be due to an increase of the pulmonary blood-pressure and to a reduced cardiac output.

The circulation time is unchanged or reduced in slightly injured animals but is prolonged in severely injured animals.

*Body Temperature in Blast Injury* (Chapter 15, page 167).

After a transient rise lasting for some 15 min after the detonation the temperature often falls and remains subnormal for the next 24 hours. This fall of temperature is believed to be due mainly to the impaired circulation.

*Changes in Blood Chemistry in Blast Injury with Special Regard to Possible Traumatic Shock* (Chapter 16, page 171).

Some of the chemical constituents of the blood have been studied in blasted animals. The decrease of haemoglobin, cell volume and plasmaprotein and the increase of plasma chlorides are shown to be a result mainly of the blood loss due to the blood sampling. The decrease of alkali reserve is supposed to be due to an insufficient gas exchange in the haemorrhagic lungs and to the deteriorated circulation. Blood sugar often increases. A slight increase has also been found of non protein nitrogen and plasma phosphorus but usually only in animals which died within some hours after the detonation. A sharp premortal increase of plasma potassium occurs. The conclusion is that the blood analyses do not speak for traumatic shock being of any particular importance in blast injury.

*Causes of Death in Blast Injury* (Chapter 17, page 183).

The direct cause of death in blast injury is a respiratory and circulatory failure. Carbon monoxide poisoning, air embolism and fat embolism have been shown to be of no importance.

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ACTA PHYSIOLOGICA SCANDINAVICA  
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*From the Institute of Physiology,  
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STUDIES ON ABSORPTION OF  
COLLOIDS AND FLUID FROM  
RABBIT KNEE JOINTS

BY

TORBJÖRN EDLUND

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## Introduction

The author will here describe methods of measuring the absorption of fluid and soluble matter from joint cavities, and the effect of various factors and drugs on the transfer of colloid and fluid through synovial membranes.

## CHAPTER I

### The Mode of Absorption of Soluble Matter from Joint Cavities in the Light of Anatomical Studies

#### Some Remarks on the Anatomy of the Rabbit Knee Joint

The literature on the anatomy of articular structures has been reviewed in detail by BAUER *et al.* (1940). HUETER (1866) is apparently the first to define the synovialis as a connective tissue structure, and this seems to be the current view (HAMMAR 1894, MARQUORT 1931, MAXIMOW and BLOOM 1942). (Although the synovialis is not a true membrane it will in the following be referred to as the synovial membrane.)

Judging by evidence presented in the literature (see BAUER *et al.* 1940 for references), the articular components embryologically and histologically seem to be of mesenchymal character, and — since the synovial lining is not a true membrane — joint cavities may be considered as tissue spaces in a somewhat modified connective tissue. No direct communication exists between the joint cavity and the blood or lymph vessels; a layer of connective tissue is always said to be interposed.

The absorption from joint cavities of various substances, especially dyes, has been studied by a number of investigators, using anatomical and histological methods. So far the observations agree as it is generally said that, after intraarticular injection of a test substance, the synovial membrane is diffusely penetrated. The further

route of removal is debated, but it seems as though both blood vessels and lymphatics are engaged in the transport of low-molecular substances; high-molecular ones involve probably only the lymphatics (see BAUER *et al.* 1940 for references).

In rabbit knee joints RIEDEL (1880) has described a tendon sheath for the extensor digitorum longus muscle — arising from the lateral condyle of the femur within the joint capsule — which was said to communicate with the joint cavity. ADKINS and DAVIS (1940) confirm these findings, and the reason may be that Riedel as well as Adkins and Davis injected larger volumes into the joint cavities, than was the rule in this investigation. The present author has not observed any penetration of test solutions into this tendon sheath (see chapter III), even when fairly high intraarticular pressures were applied. EFSKIND (1941 b) was also unable to find any communication between the joint cavity and this tendon sheath.

## CHAPTER II

### General Methods

1. *Test animals.* Apparently healthy, male rabbits weighing  $2.0 \pm 0.1$  kg were used throughout. Animals with signs of skin lesions were discarded. The animals were given hay, oat and water ad lib., and were not used earlier than 48 hours after arrival from the breeders. In the meanwhile they were caged, the floor space not exceeding  $100 \times 100$  cm.

2. *Anesthesia.* 6 ml per kg body weight of a 25% urethane solution was employed and given intravenously during 15—20 minutes in one of the marginal ear veins. In all the experiments the body temperature was maintained at a normal level by means of electrically heated operating tables. Animals which moved their legs during the experiments were discarded as well as all other experiments with evident technical errors.

3. *Hemoglobin solutions.* Human hemoglobin was used throughout. Three times washed blood corpuscles were hemolyzed with twice the corpuscle volume of distilled water, 2 g of NaCl per 100 ml was added to the hemolysate, then the ghosts were removed by centrifuging. After dialysis against running tap water for 16—18 hours (temperature of tap water 7—10° C.) the hemoglobin concentration was determined in the dialyzed fluid, freshly distilled water was added to the solutions so that the hemoglobin concentration became 5.5 g per cent, to each 100 ml of such solutions was added 0.015 moles NaCl. The solutions were sterilized immediately by filtering through a Seitz' filter and stored at  $-0.5^{\circ}$  C. The solutions were used within 14 days after sterilization

and withdrawn from the bottles under aseptical conditions. Such solutions have a pH of 7.3—7.4. Their freezing point depression is  $-0.58^{\circ}\text{C}$ . 4 random samples from different preparations gave the following analytical values: Sodium (determined with a Perkin Elmer Corp. flame photometer Model No. 52 A), 146, 148, 147, 148 millimoles. Potassium (flame photometer) 0.7, 0.8, 0.5, 0.7 millimoles. Calcium (determined according to KRAMER and TRSDALL's method) 15.2, 15.7, 16.2, 15.1 mg per cent. (The calcium contents came from calcium salts in the tap water used in the dialyzing procedure.) Chlorides (determined by electrometrical titration with  $\text{AgNO}_3$ ) 150, 149, 148, 152 millimoles.

4. *Isotonic electrolytic solutions* used as test fluids in the absorption or perfusion experiments. These solutions were prepared from freshly distilled water and stored at  $-0.5^{\circ}\text{C}$ . In chapter V there is used a solution, equiionic with the hemoglobin solutions, it contains 150 millimoles of  $\text{NaCl}$  and 15 mg per cent of calcium (as  $\text{CaCl}_2$ ).

5. *Staphylococcus strains*. The strain<sup>1</sup> used was an  $\alpha$ -hemolyt., coagulase and hyaluronidase producing strain of *Staph. aureus*. Throughout, the nutrient medium was ascitic broth. The 24-hour cultures mentioned below were prepared from cultures contained in ampoules, evaporated in vacuum and filled with nitrogen before sealing. The ampoules were stored at  $-20^{\circ}\text{C}$ . The contents of the ampoules were dissolved in some drops of the ascitic broth, these drops were inoculated in the medium and after incubation for 24 hours at  $37^{\circ}\text{C}$ . the culture was ready for use.

6. *The hemoglobin concentrations* in the tested solutions were determined by WU's method (1922) as modified by PETERS and v. SLYKE (1932). The alkaline hematin was determined in a Pulfrich photometer, filter S 50, 1 cm cuvettes. Lambert-Beer's law is valid over the entire concentration range used. The standard error of a single determination is, expressed as the coefficient of variation,  $\pm 3.6$ ,  $\pm 1.5$  and  $\pm 0.7$  per cent for extinctions of respectively 0.122, 0.267 and 1.040. The extinction/g per cent is 0.0447 when 0.05 ml of hemoglobin solution is added to the reagents, and it is the same when 0.05—0.4 ml of the solutions are added to the reagents and the measured extinction values are corrected to a volume of 0.05 ml of the hemoglobin containing samples. WU's statement that plasma proteins and stroma constituents do not interfere with the measurements was verified. The same was true when human synovial fluid was added to an equal volume of a hemoglobin solution. The extinction/g per cent hemoglobin was obtained by means of determinations of the oxygen capacity of human blood and checked by determining the iron in hemoglobin solutions according to HANZAL's (1933) method.

7. *Insertion of needles* into the knee joints of the rabbits. Blunt needles (length 18 mm, external diameter 0.7 mm) were inserted through the patellar tendon after making a transversal incision in the skin about half way between

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<sup>1</sup> I wish to thank the members of the scientific and technical staffs of the Institute of Bacteriology, Uppsala, who have suggested the procedure used of preparing the ampoules and carried out the technical work with the cultures.

the upper termination of the tibia and the distal termination of the patella. To eliminate leakage sutures were stitched round the needles and finally the skin was sutured. In the experiments to be described only knee joints were used.

8. *Temperature of test solutions.* All test solutions used as indicators in absorption tests were warmed to 38° C. before being injected intraarticularly.

9. *Statistical methods.* Ordinary standard methods were used. The significances of differences were calculated according to the formulae for small samples given in YULE and KENDALL (1946), chapt. 23. Differences were considered significant when the probability that they were accidental was less than 0.01. The average correlation coefficient of individual experiments in the same group was calculated according to SNEDECOR (1946), p. 151. Correction was made for bias in  $z$ . Regression lines calculated according to the method of least squares on total samples were tested for linearity according to the method given in WEATHERBURN (1946), p. 225.

## CHAPTER III

### The Influence of Pressure on the Flow of Fluids through Synovial Membranes

“With the aim of determining the structural conditions which affect fluid movement through the cutaneous connective tissue of mice”, MC MASTER (1941 a, b, c) employed a method whereby various fluids were brought into contact with the tissue. The needle carrying fluid from the perfusion apparatus was inserted into the tissue in such a manner as to make impossible direct liquid entry into blood vessels or lymphatics. MC MASTER demonstrated that, at atmospheric pressure, Locke’s solution entered the tissues intermittently. At raised pressures — more than 4.5 cm H<sub>2</sub>O — the flow became continuous but the rate did not increase significantly until the pressures were raised above a mean of 8.5 cm H<sub>2</sub>O. At this latter pressure the resistance to flow of the tissue suddenly became markedly smaller as though the homogeneity of the interfibrillar matrix had been broken. At this and higher pressures the inflow increased greatly at a rate directly proportional to the pressure applied. Other fluids that partly remained unresorbed at atmospheric pressure, viz. homologous serum and pontamine sky blue dissolved in Locke’s solution, gave identical results with respect to the “breaking point”.

The pressure at which the inflow changed — here and by Mc MASTER called the “breaking point” — proved to be independent of the circulation (found in dead animals, Mc MASTER 1941 c). Mc MASTER concluded that in normal connective tissue the movement of fluid did not follow preformed channels; above the breaking point spaces were forcibly broken up. The intermittent inflow of Locke’s solution was attributed to “vasomotion”<sup>1</sup> of the blood capillaries (for review of the literature cf. CHAMBERS and ZWEIFACH 1947). These findings confirm previous observations relating to the perifibrillar movement of dye solutions in normal connective tissue where the fibrils serve as conductors (Mc MASTER and PARSONS 1939, a, b). These authors suggest that in connective tissue dye movement takes place in thin perifibrillar films where the liquid is firmly held to the fibres by surface forces. These adherent films of liquid were supposed to be demonstrated by the use of dyes and found unable to move freely. They were considered to be so thin that practically they were part of the connective tissue and not interstitial pools of fluid. In other words there is no freely movable liquid in normal connective tissue; it is homogeneous. As mentioned in the foregoing (p. 5) joint cavities may be regarded as connective tissue spaces. It seems desirable to determine the breaking point, if any, of the synovial membrane before continued studies are performed on the absorption from joints, under physiological structural conditions.

The following method, based on the same principles as Mc MASTER’s, was adopted to prove the existence of a breaking point in the synovial membrane.

### Method and Apparatus

Perfusion apparatus, see fig. 1.

A 2 ml burette J, graduated in 0.01 ml, each division occupying a length of 1.80 mm, is carried on a horizontal panel F. A spirit level G is used to check the horizontalness of F. The burette J is connected to a water manometer L and a T-tube N via rubber tubings and an ordinary suction flask K. The perpendicular arm of the T-tube is immersed in water contained in the vessel M. The horizontal arm of N is connected to an oxygen supply. The pressure of the oxygen on the water column in J and the water manometer L can be varied at

<sup>1</sup> For definition of the term see page 52 of the present paper.

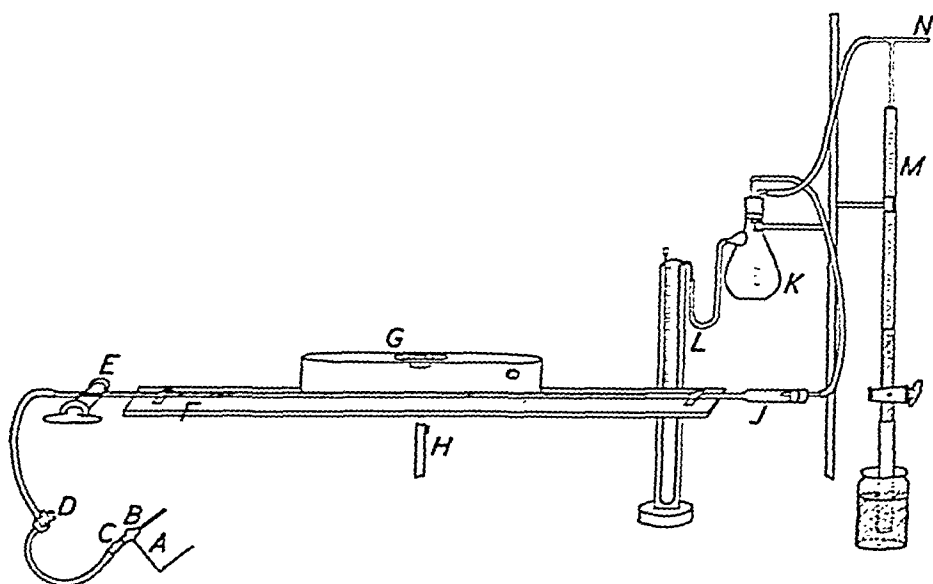


Fig. 1. Perfusion apparatus. For details see text.

will within the range 0—32 cm of  $H_2O$  by adjusting the level of the water column in M. The delivery tube of the burette is by a flexible tube joined to the three-way stopcock D, one arm of which is fitted to the needle holder of an ordinary hypodermic syringe. Another arm of the stopcock is flexibly connected to the injection needle B. C is a stainless steel adaptor fitted in the socket of B. In carrying out the perfusion experiments the burette must be adjusted to the same level as the point of the intra-articularly inserted needle B. This is accomplished by means of the right-angle wire A, one member of which is so mounted on the socket of the needle as to make the other member parallel therewith. When the anchored arm of A is horizontal the point of the needle will be in level with the free arm of A. The burette is positioned by means of the stand H (constructional details illustrated in fig. 10 p. 25).

### The Perfusion Experiments

The burette and the needle assembly were connected up and filled with mammalian Ringer solution through the free arm of the stopcock D so that no air remained between the point of B and the free liquid surface in J. The communication between B and J was then broken by turning the stopcock E.

The anesthetized rabbit was placed on a small electrically heated operating table. The hind legs of the rabbit were tied down (see fig. 29 b p. 103). The needle having been inserted in the joint cavity (details p. 7), the perfusion experiment was carried out as follows:

The point of the needle and the burette were first put in level. Then 0.5 ml of Ringer solution was injected into the joint cavity through the free arm of D. The pressure on the water column in J was adjusted to about 3.0 cm of  $H_2O$  by means of the pressure regulator M. Communication with the joint cavity was established by turning the stopcock E and the perfusion experiment was under way. That there actually was connexion between the joint cavity and the burette was determined by applying gentle pressure on the suprapatellar recess whereby the fluid meniscus in J moved up.

The inflow rate was measured from about 3.0—9.0 cm of  $H_2O$  at approximately 1 cm intervals. Above 9.0 cm an interval of 3 cm was used.

Experience gained from preliminary experiments had demonstrated that below 9.0 cm of water 2 minutes and above that figure 4 minutes were adequate to compensate for the volumetric expansion of the joint cavity specifically due to the increase in pressure.

### Results

20 rabbits were used. In 13 of these the inflow rate was observed for 10—12 minutes at each pressure level and in 7 for 4—6 minutes. In each rabbit one knee joint only was used. The average flow in ml/min. at each pressure level was calculated. The constancy<sup>1</sup> of flow was checked by taking intermediate readings every second minute. The average flow figures ( $F$  ml/min.) thus obtained were plotted graphically as a function of the pressure ( $P$  cm  $H_2O$ ).  $F = f(P)$ .

Essentially the curves were similar to Mc MASTER's, i.e. the synovial membrane apparently has a breaking point.

The two parts of the straight linear curves were calculated by the method of least squares and the point of intersection was registered as the pressure level of the breaking point.

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<sup>1</sup> As was to be expected the inflow was not intermittent. In such a large aggregate of tissue as the synovial membrane the vasomotion, if any, per unit of time should be statistically constant. Moreover, the intermittency reported by Mc MASTER is more pronounced at lower pressures than those used here.

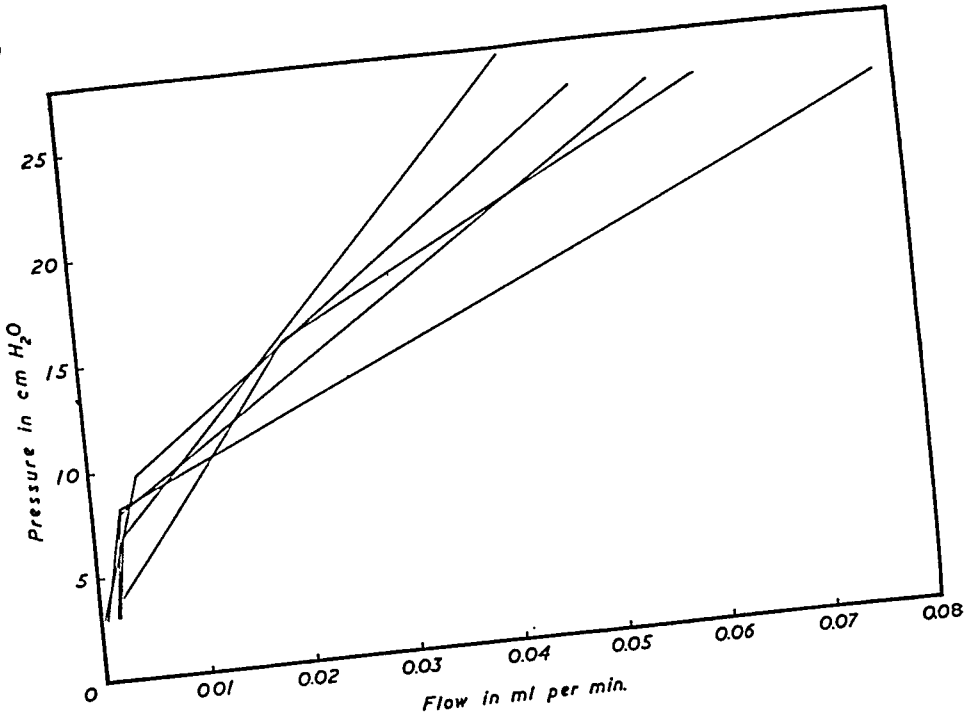


Fig. 2,

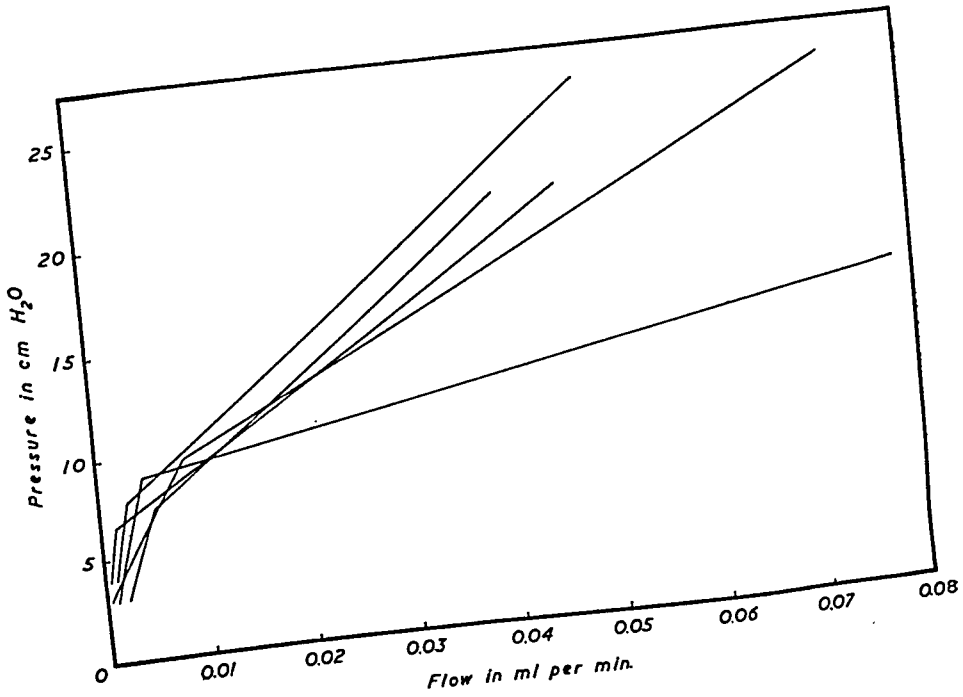


Fig. 3.



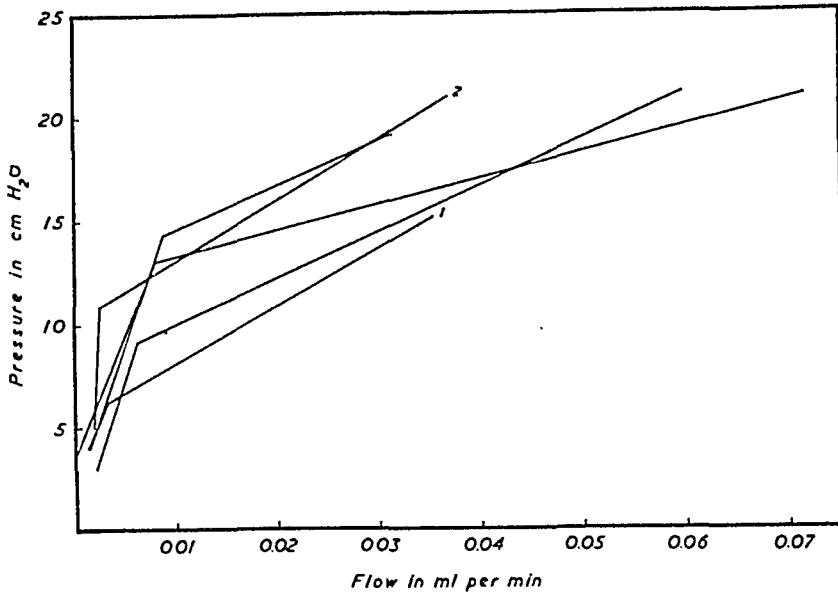


Fig. 4.

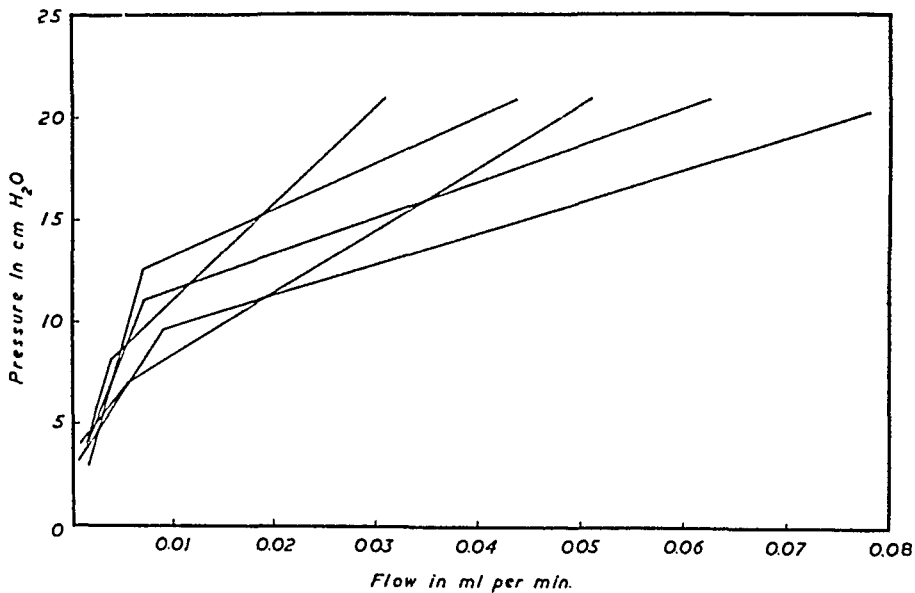


Fig. 5.

Fig. 2, 3, 4, 5. Initial perfusion pressure about 3.0 cm H<sub>2</sub>O. Curves start at pressures where measurable inflow first could be registered and end at the highest used pressure. In fig. 2 and 3 experiments with 10–12 minutes perfusion at each pressure are shown. Curves marked 1 and 2 in fig. 4 are experiments where perfusion periods of 4–6 minutes were used, the other curves in fig. 4 are from experiments with 10–12 minutes perfusion periods. In all experiments shown in fig. 5 perfusion periods of 4–6 minutes were used.

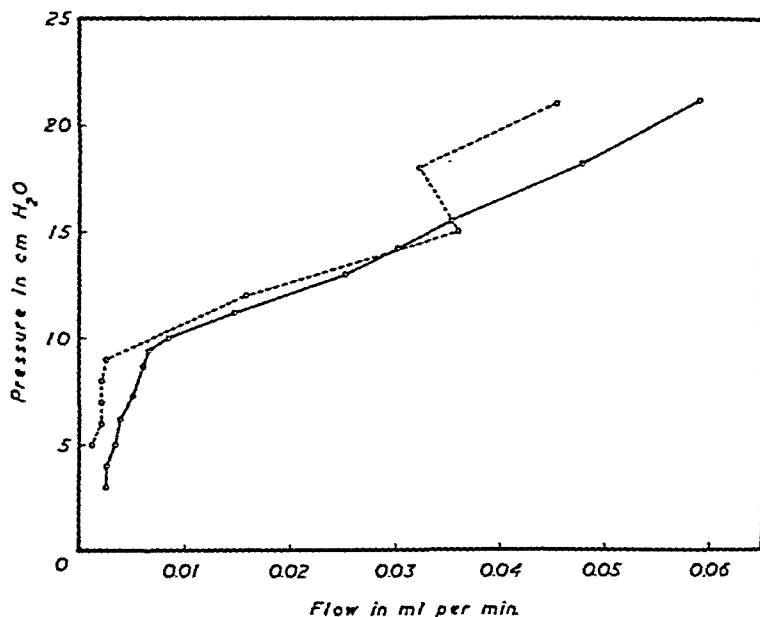


Fig. 6. A typical (full line) and the least successful (dotted line) experiment chosen from curves shown in fig. 2, 3, 4, 5. Open circles indicate perfusion pressure levels and corresponding flow rates.

There was no difference between the two sets of experiments, viz. those with 10—12 min. perfusion at each pressure, and those with 4—6 min. perfusion. See figs. 2, 3, 4, 5 and table 1.

A typical experiment and the least successful one are illustrated graphically in fig. 6.

From table 1 it appears that the mean breaking point is  $9.55 \pm 0.58$  cm H<sub>2</sub>O ( $\pm$  standard error of the mean).

*Experiments concerning the relation between pressure and rate of flow when the breaking point is exceeded by the initially applied perfusion pressure.*

In conditions of pronounced inflammatory edema MC MASTER (1941 c) found no breaking point. The inflow was directly proportional to the pressure and increased greatly for a slight increment in pressure. This was interpreted as being due to free movement of liquid in channels preformed by the formation of edematous fluid which had broken the homogeneity of the matrix of the connective tissue of the skin. That in edema the captured perifibrillar films are actually changed into free movable pools of tissue fluid, was shown by MC MASTER and PARSONS (1939 a, b). This being so, the application

TABLE 1.

Type of experiment	Perfusion period at each pressure level	Breaking point in cm H <sub>2</sub> O	$\frac{dF}{dP}$ for pressures below breaking point ml/min/cm · 10 <sup>5</sup>	$\frac{dF}{dP}$ for pressures above breaking point ml/min/cm · 10 <sup>5</sup>
I. Normal synovial membrane. Initial perfusion pressure about 3 cm H <sub>2</sub> O 13 animals	10—12'	$m = 9.66$ $e = \pm 0.79$	$m = 64$ $e = \pm 10.0$	$m = 427$ $e = \pm 70$
II. Normal synovial membrane. Initial perfusion pressure about 3 cm H <sub>2</sub> O 7 animals	4—6'	$m = 9.33$ $s^2 = 4.88$	$m = 85$ $s^2 = 2592$	$m = 408$ $s^2 = 21894$
Combined mean of I and II				
		$m = 9.55$ $e = \pm 0.58$	$m = 71$ $e = \pm 9.5$	$m = 421$ $e = \pm 49$
III. Normal synovial membrane. Initial pressure above breaking point 6 animals	4—6'			$m = 265$ $s^2 = 12977$
IV. Exsanguinated rabbits 5 animals	4—6'	$m = 9.29$ $s^2 = 8.38$	$m = 56$ $s^2 = 320$	$m = 408$ $s^2 = 21414$
V. Normal synovial membrane. Coloured isotonic sodium iodide solution 5 animals	4—6'	$m = 9.04$ $s^2 = 6.94$	$m = 93$ $s^2 = 1643$	$m = 367$ $s^2 = 18190$

Signs:  $m$  = arithmetic mean  
 $e$  = standard error of the mean  
 $s^2$  = variance

of an initial perfusion pressure well above the breaking point of the synovial membrane should destroy the homogeneity of the inter-fibrillar matrix of the synovial membrane. If the experiment were

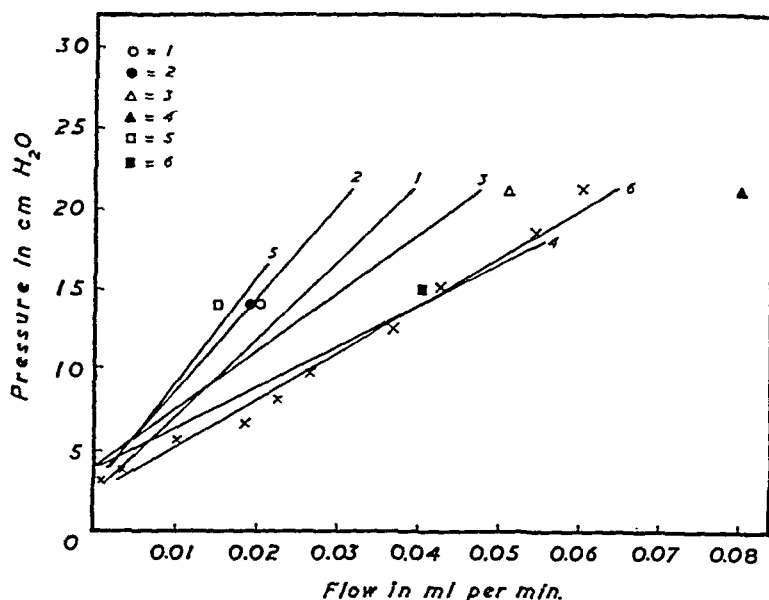


Fig. 7. High initial perfusion pressure level above the "breaking point". Signs indicate the initial perfusion pressure and corresponding numerals the curves belonging to the respective signs. In order to give an idea of the accuracy of the method perfusion pressure levels and corresponding flow rates in experiment 6 are indicated by crosses. Average correlation coefficient calculated from the correlation coefficient of each line and used as a measure of linearity = 0.983. Range of correlation coefficients for the six experiments = 0.993—0.908. For method of calculation of the average correlation coefficient see p. 8.

afterwards resumed in the manner described above at a pressure of about 3.0 cm  $H_2O$  the proportion between inflow and pressure would be constant at all the applied pressures. Such was actually the case. See fig. 7 with caption and table 1. In the experiments with high (breaking) initial pressures the inflow was determined during 4—6 minutes at all the pressure levels used.

### Controls

The following factors must be discussed before these results can be taken at face value:

1. *The sensitivity of the perfusion apparatus.* The least pressure required to cause noticeable flow through the apparatus was measured with a micromanometer according to Mc MASTER's method (1941 a). The needle B was horizontally submerged just below the surface of the liquid in a large Petri dish filled with Ringer, the burette being in

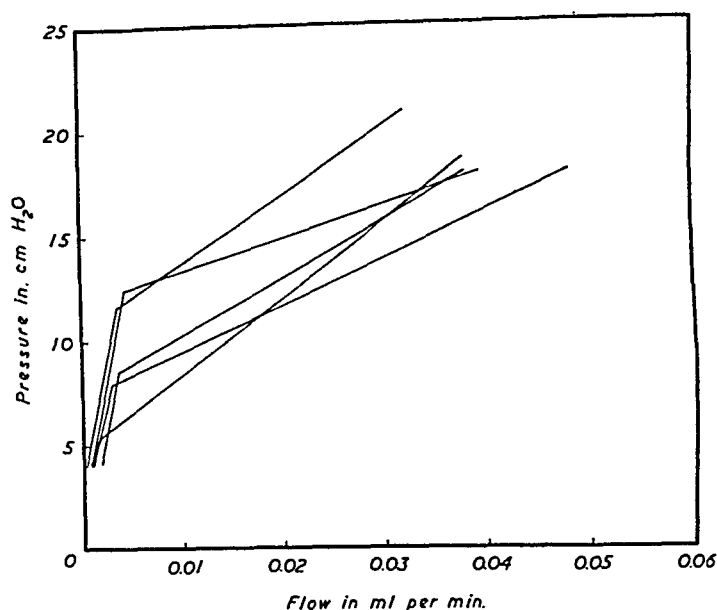


Fig. 8. Exsanguinated animals. Curves start at pressures where measurable inflow first could be noticed and end at the highest pressure used.

the same plane as the liquid surface in the dish. The necessary pressure was 0.1–0.15 cm of  $H_2O$ , and can thus be neglected.

2. *Internal friction in the apparatus.* Having filled the burette with Ringer the internal friction was measured to correspond to a pressure drop between the manometer L and the point of the needle B of 6.6 cm of  $H_2O$  at a flow of 1.0 ml/min. and as could be expected it was directly proportional to the flow. Obviously this internal friction can be neglected for the flows found in these perfusion experiments.

3. *Circulatory phenomena as the possible cause of the breaking point.* 5 rabbits were anesthetized as before and killed by exsanguination. When the heart had stopped beating the perfusion experiments were carried out. In all cases a breaking point was found (see fig. 8 and table 1).

4. *An enlargement of the articular absorptive surface as the cause of the sudden increase in flow.* As described on page 6 RIEDEL has (in rabbits) described a tendon sheath which communicates with the joint cavity and possibly this sheath might open at a certain pressure, drawing away liquid from the joint cavity and simultaneously enlarging the absorptive area. In order to obviate this possibility the following experiments were devised. 2.5 per cent sodium iodide

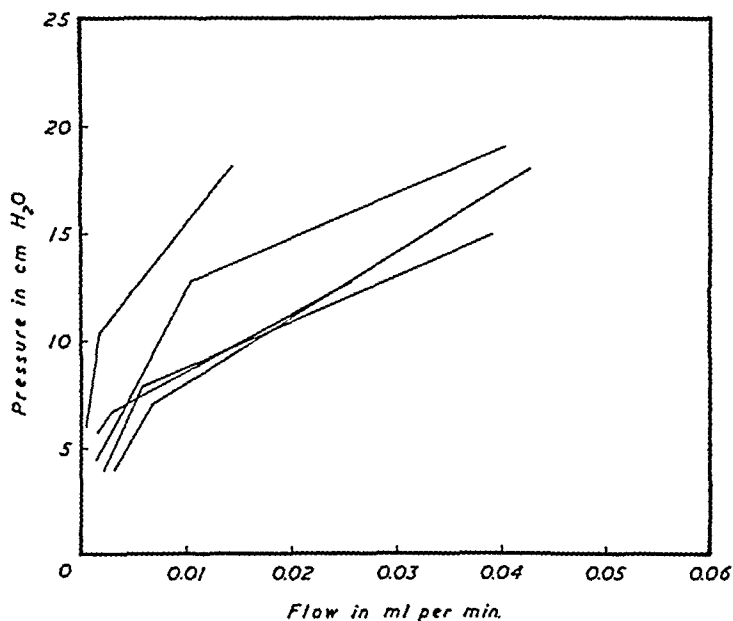


Fig. 9. Perfusion fluid: coloured sodium iodide solution. Curves start at pressures where measurable inflow first could be noticed and end at the highest pressure where the inflow rates were determined.

solution containing 10 mg per cent of calcium ( $\text{Ca Cl}_2$ ) coloured with Chlorazol sky blue<sup>1</sup> (0.5 per cent) was perfused and here also a breaking point was found (see fig. 9). After each experiment the perfusion pressure into the joint was raised to 32.0 cm  $\text{H}_2\text{O}$  for 10 min., whereupon a tourniquet (rubber tubing, 4 mm ext. diam.) was tightly applied on the foreleg immediately below the upper termination of the tibia. The site of the tendon sheath was explored and in no case could the presence of any dye be detected. Examination of the puncture wounds showed that no dye had penetrated into the patellar tendons. The increased flow at pressures above the breaking point cannot well be due to an enlargement of the joint cavity proper since, if such were the case and all other absorptive conditions being equal, the area would on the average have become about 6 times larger (see table 1).

Moreover, a closer examination of X-ray photos taken by SIGURDSON (1930) show that 0.5 ml of lipoiodol completely filled the joint cavity in a animal material similar to the present one. The same has been found by the present author (see fig. 30 p. 104).

<sup>1</sup> Colour index 1924 no. 518.

### Comment

By a method similar to MC MASTER's the author studied whether or not the synovial membrane has a breaking point. MC MASTER's delicate method of preventing the perfusion needle from direct contact with blood or lymphatic vessels was rendered unnecessary by choosing another point of attack, viz. the needle was inserted into a ready-made space in connective tissue, the knee joint.

The breaking point of the synovial membrane found by these experiments is of the same order of magnitude as that in dermal connective tissue found by MC MASTER in his perfusion studies.

It was conclusively established by studies on exsanguinated dead animals that both the breaking point and the general course of the "pressure/perfusion" curve are independent of the circulation.

The inflow rate was found to be directly proportional to all applied pressures when these followed an initial perfusion pressure well above the breaking point. This was interpreted to mean that the high initial pressure resulted in the introduction of freely movable fluid into the tissue which in turn decreased the resistance to flow of the synovial membrane by enlarging its "pores".

Experiments with infusion periods of 4—6 min. instead of 10—12 min. demonstrated that the breaking point was independent of the duration of the liquid inflow.

### Discussion

MEYER and HOLLAND (1932) and HOLLAND and MEYER (1932) concluded that fluid flow in normal dermal connective tissue in man obeyed Poiseuille's law for viscous flow through a capillary tube, i. e. took place in preformed channels. They attributed their findings to the existence of a freely movable tissue fluid. Furthermore, these authors by extrapolations to zero flow assumed they had determined the tissue pressure in human, normal and edematous (cardial and inflammatory edema), connective tissue.

As MC MASTER points out (1941 c) they must have initiated their perfusion experiments at pressures well above the breaking point. Taking for granted the existence of a breaking point also for human dermal connective tissue, the present author assumes that they, for the part of the curve above the breaking point, extrapolated the press-

ure causing zero flow, their "tissue pressure". They found a lower tissue pressure in edematous than in normal skin, probably by extrapolating to zero flow along straight lines similar to the ones obtained in the present author's experiments (see above) with initial high pressures. This interpretation is justifiable since adequately measured tissue pressures are approximately equal or higher in edematous than in normal tissue (MC MASTER 1946 a). Consequently the method used by HOLLAND and MEYER does not warrant any conclusions as to the movement of fluid or the pressure in normal connective tissue.

*The relation between pressure and rate of flow at pressure levels below the breaking point.*

In the present study on joint cavities the flow at pressures below the breaking point increased significantly as the pressure was raised:  $\frac{dF}{dP} = (71 \pm 9.5) 10^{-5}$  ml/min/cm (see table 1). MC MASTER (1941 c) reports that below the breaking point the rate of flow was not significantly influenced by increases in pressure.<sup>1</sup>

It is impossible to decide whether this discrepancy between MC MASTER's and the author's findings is due to any functional or anatomic difference between the dermal connective tissue of mice and the synovial membrane of the rabbit.

However, since dermal connective tissue and the synovial mem-

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<sup>1</sup> Assuming that in the above experiments the increased flow is not influenced by increments in pressure but caused by an enlargement of the absorbent area in the joint — the properties of which are considered constant — the 20 experiments permit the following evaluations. Calculated mean flow at 5 cm H<sub>2</sub>O pressure =  $0.0023 \pm 0.00017$  ml ( $m \pm em$ ). Calculated mean flow at the breaking point =  $0.0058 \pm 0.00090$  ml ( $m \pm em$ ).

Since the ratio between these values is 2.5 the absorbent area should have increased 2.5 times, which is improbable.

It may furthermore be said that the augmentation of flow as the pressure increases — in the author's experiments — is due to a slight edema caused by the instilled Ringer solution. This would be in line with MC MASTER's findings (1941 c) in early inflammatory edema where he demonstrated the existence of a breaking point and a rate of flow dependent on the pressure at all pressure levels. However, if the above significant  $\frac{dF}{dP}$  is induced by an edema which in its turn is caused by the perfusion fluid, the magnitude of  $\frac{dF}{dP}$  in the experiments with 4—6 min. perfusion ought to differ from that in experiments with 10—12'.



brane may be looked upon as dead systems<sup>1</sup> D'Arcy's law<sup>2</sup> of fluid flow through granular beds or some of its extensions should be applicable provided that the limitations imposed by the changes in pore diameters and the deformation of the tissues caused by the increased pressure are kept in mind.

Of deformable media it is known that the permeability decreases as the pressure drop across the bed increases, especially when the particles forming the bed are easily deformable. (UNDERWOOD quoted from CARMAN 1937). The decrease in permeability is certainly not caused by change from viscous to turbulent type of flow (CARMAN 1933). On the other hand an increased permeability is sometimes caused by an increased pressure drop across filter cloths, and UNDERWOOD attributed the former to stretching of the fibres, thereby enlarging the pores in a cloth supported on a series of ridges.

Thus, the possibility exists that at pressures below the breaking point the almost constant rate of flow reported by Mc MASTER (1941 c) may be due to a deformation of the connective tissue which reduces the permeability by decreasing the pore diameters.

The laws governing fluid transfer through deformable media applied to fluid passage in connective tissue (considered as a dead system) suggest that the above described discrepancy between the author's and Mc MASTER's findings may be explained by differences in the "ridges of the filters", i.e. differences in the fibrillar structures of dermal connective tissue and synovial membranes.

However, the above mentioned limitations of D'Arcy's law, together with others not mentioned here (ERIKSSON 1920, BOZZA and SECCHI 1929; for discussion of these papers see CARMAN 1937, SULLI-

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<sup>1</sup> A simplification of this kind can be justified by the similarity in dead and living animals of experimental perfusion findings.

<sup>2</sup> D'Arcy's law, deduced empirically from the flow of water through sand and sandstone, can be expressed by the equation:

$$u = \frac{K \times \Delta P}{L}.$$

$u$  = volume rate of flow through the bed;  $K$  = Coefficient of permeability; (Rate of flow of water across a unit cube of the sand at unit pressure head.)  $\Delta P$  = Pressure drop across the bed;  $L$  = depth of the bed; (Quoted from CARMAN 1937). For bibliography of fluid flow through granular beds see CARMAN (1937), SULLIVAN and HERTEL (1942), where the various extensions are listed and discussed.

VAN and HERTEL 1942) indicate the uselessness of further discussions, only based on perfusion experiments, of the state of tissue fluid or of the effect of pressure on pore diameters and thereby on permeability.

*The relation between pressure and rate of flow at pressures above the breaking point.*

When the perfusion pressures were raised above the breaking point the average  $\frac{dF}{dP}$  increased about 6 times (table 1). A statistically not significantly different mean  $\frac{dF}{dP}$  was obtained in the experiments with high initial perfusion pressures.

These results agree well with those of MC MASTER (1941 c), i.e. the resistance to flow of connective tissue decreases above the breaking point. Probably the decrease is caused by forced induction of free movable fluid into the tissues, leading to an enlargement of the tissue "pores". The perfusion experiments with high initially applied pressures indicate that once induced this decreased resistance to flow of the synovial membrane is in evidence also at pressures below the breaking point. This means that the separation of the tissue elements is not immediately reversed by the elastic forces of the tissue.

### Conclusions

Since joint cavities may be taken as being connective tissue spaces (see p. 5) results and interpretations from investigations into fluid movement in dermal connective tissue should be at least partly valid for the synovial membrane. By means of the method adopted in the present investigation similarity has been established with respect to the magnitude and existence of a breaking point, which is independent of the blood circulation. Furthermore, it has been demonstrated that initial perfusion pressures above the breaking point decrease the resistance to flow when subsequently lower perfusion pressures are applied. This shows that elastic forces are not immediately able to restore the tissue to the normal state when an internal derangement once has been caused by an introduction of fluid, and this is interpreted as being due to an artificial edema. It is impossible to decide whether the statistical identity of the breaking point values for dermal

connective tissue in mice and of the synovial membrane in rabbits are attributable to a general characteristic of connective tissue.

It must be stressed that the author's method and experiments only deal with the resistance to flow of the synovial membrane. They give no information about other forces than the intraarticular hydrostatic pressure as causing fluid movement through the synovial membrane.

In the view of the present author perfusion experiments yield no adequate information as to pore diameters and state of the tissue fluid, the reason being the complicated manner in which liquids flow through deformable media.

The findings related above make it clear that continued research into the absorptive properties of normal joints must be carried out at intraarticular hydrostatic pressures below the breaking point if the normal structure of the synovial membrane is to be preserved during the experiments. The ideal pressure level for such studies would be the "tissue pressure" of the synovial membrane which, however, never has been measured.

## CHAPTER IV

### Methods for Determinations of Intraarticular Hydrostatic Pressure and Absorption of Colloid and Fluid from Joint Cavities

#### The Influence of Intraarticular Hydrostatic Pressure and Changed Structural Conditions of the Synovial Membrane on Absorption

In chapter III it was shown that in rabbit the synovial membrane has a breaking point of the same order of magnitude as that of dermal connective tissue in mice. The importance of knowing the initial hydrostatic pressure of the test solution was also demonstrated; absorption tests at initial pressures above the breaking point being unable to adequately illustrate absorption through a structurally intact membrane.

The graph given by SMITH and CAMPBELL (1929) suggests that joint flexion alters the intraarticular hydrostatic pressure induced by

a given amount of test fluid in the knee joints of cats. If this were so, the possibility exists that equal quantities of intraarticularly injected test solution can cause significantly different initial hydrostatic pressures and pressure/time curves, depending on the degree of joint flexion in diverse groups of rabbits. This would imply that the effect of the hydrostatic pressure on joint absorption may be measured at pressure levels that, within limits, can be changed at will.

It would also be interesting to study the effect of the intraarticular hydrostatic pressure on absorption from joints at pressure levels below the breaking point when the structure of the synovial membrane has been deranged by other means than the introduction of fluid at sufficient pressure.

The following methods were developed to render possible an investigation into the above matters:

1. *A method of determining the intraarticular hydrostatic pressure.*
2. *A method of deranging the structure of the synovial membrane without introducing intraarticular fluid.*
3. *A method of finding the amount of colloid and fluid absorbed from knee joints in rabbits.*

## Part I

### Measuring the Intraarticular Hydrostatic Pressure and Deranging the Structure of the Synovial Membrane

#### Method and Apparatus

The apparatus (see fig. 10) consists of a microscope tube C carrying a rectangular lucite plate D with a lucite stud projecting coaxially from each short end. A cylindrical bore with diameter 1.0 mm runs from stud to stud through D at right angles to the optical axis of C. One of the studs is connected to a three-way stopcock E by rubber tubing. The opposite stud is taper ground to fit the socket of an ordinary hypodermic needle. By means of the T-tube F the bore through D is indirectly connected to a water manometer G, and a bulb of thick rubber placed in the screw press A. The microscope C is so mounted on the stand B as to be rotatable about its own axis and universally movable by means of the articulated joints of the stand. C is provided with a micrometer eyepiece with 50 scale divisions, each cor-

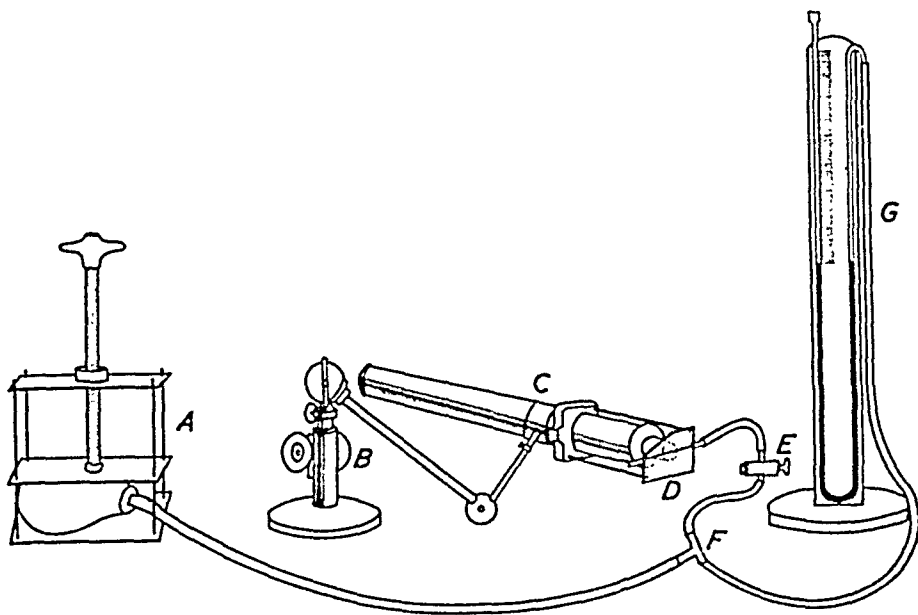


Fig. 10. Apparatus for measurements of intraarticular hydrostatic pressure.

responding to 0.01 mm. The microscope C is permanently focussed on the bore through D. All connections in the assembly are air-tight.

**PREPARING THE APPARATUS.** Immerse the free stud on D in kerosene so that a minimum quantity enters the capillary bore. Draw up under ocular control through the microscope enough test fluid in the capillary to bring the kerosene meniscus to the midpoint of the scale in the micrometer eyepiece when the capillary below the midpoint is completely filled with liquid. Turn the stopcock E so that no connection exists between D and A and G.

**MEASURING INTRAARTICULAR PRESSURES.** As before the animals are anesthetized and, the hind legs having been secured in the desired position, a needle is inserted in the knee joint (p. 7). The free stud on D is carefully positioned to fit the socket of the needle by means of the stand B. By means of a rack and pinion arrangement on the stand the stud is then temporarily removed from the needle socket to allow an injection of test fluid to be made. Before starting the experiment it should be doubly ensured that there is no air between the test fluid in the joint and that in the capillary through D by drawing up fluid into the capillary from the joint after making the connection. (The volume of the capillary above the micrometer scale by far exceeds the sum of the volume of the bore in the needle and

the volume of any unfilled socket space.) This is easily brought about by exerting pressure on the rubber bulb in the screw press A and thereby adjusting to an adequate level the pressure on the water column in G and the test fluid meniscus in D.

By adjusting the pressure level as described above the meniscus in the micrometer eyepiece is constantly kept at the midpoint during the test period. Only those experiments were considered satisfactory in which there was free connection between the registering system and the joint cavity and this was considered to be the case when the meniscus in D could be seen to slowly oscillate in tempo with the respiration of the animal.

The pressure required to hold the meniscus at the midpoint of the scale in the micrometer eyepiece was measured by the water manometer G. At the beginning of each experiment readings were usually taken every minute, later every two minutes.

The experiment having been concluded, the animal was carefully removed from the table so that the microscope assembly and the needle retained their respective positions and were in no way jarred. The "negative pressure" required to force test fluid to the midpoint of the micrometer scale was then measured by immersing the point of the needle just below the liquid surface in a small flask containing test fluid. The "negative" pressure thus obtained was subtracted from the previously obtained pressures, the result being the pressure exerted in each experiment at the point of the needle. The pressure measuring equipment is accurate within  $\pm 1.0$  mm H<sub>2</sub>O.

#### **Method of Deranging the Structure of the Synovial Membrane**

It is well known (WHITE, *et al.*, 1933) that muscular movement greatly increases the flow of lymph from the moved part. The net transfer of fluid from the capillaries into the tissues has in other words increased. Consequently, it was surmised that muscular movement of the hind legs of rabbits might so greatly increase the outflow of fluid from the blood vessels that the repercussions on the synovial membrane could be similar to those found in the previous chapter where initial "breaking pressures" were applied, i. e. the synovial membrane would be structurally deranged by pools of free movable liquid in the tissues. In order to determine whether previous movement of the legs caused the same type of pressure/flow curves as those

found in edematous tissue (Mc MASTER 1941 c), the following method was adopted.

The animals were exercised in a tread-mill for 4 minutes. The endless belt moved at a rate of 46—47 m per min. After 2—3 hours the procedure was repeated.

Joint perfusion experiments were carried out as described in chapter III 17—18 hours after the last run, the perfusion period at each pressure being 4—6 minutes.

It may be mentioned already here that, judging by the respiration rate after the exercise, the untrained rabbits used in the present investigation became fairly exhausted by such a run. However, the exhaustion was never sufficient to render the animals unable to keep away from the induction coil placed at the rear of the treadmill.

#### Measurements of Intraarticular Pressure at Various Degrees of Joint Flexion

The following degrees of flexion from the extended position were used, viz. 0°, 60°, 90°.

These angles were checked by set squares, the ventral muscles on the thigh and foreleg serving as lines of reference. The different degrees of flexion were obtained by varying the manner of binding down the hind legs. In tying down the fully extended legs just enough force was used to cause the desired flexion. At each degree of flexion the extent of abduction appears in figs. 29 a, b, c, p. 103, which require no comment.

**TEST FLUID.** 0.85 ml of a 5.5 g per cent hemoglobin solution (for data on such solutions see p. 7) was injected intraarticularly into each animal and the pressure measuring apparatus was assembled as described above. The pressures were registered during 40 minutes and after the before mentioned correction they were considered to indicate the intraarticular hydrostatic pressure. Since it took some time to adjust the apparatus and see that the oscillations of the meniscus kept time with the rate of respiration of the animal, the first pressure could be measured 1 min. after the injection of test fluid. One knee joint only was used in each animal.

**CLASSIFICATION OF THE ANIMALS TESTED.** Three groups of animals were used, the respective degrees of flexion being 0°, 60°, and 90°.



One group of animals had been subjected to a previous run in a tread-mill as described above. In this group the degree of flexion was 60°.

### Results of Intraarticular Hydrostatic Pressure Measurements

Rectilinear curves were obtained in all the experimental groups by plotting the registered pressure in cm H<sub>2</sub>O as a function of the square root of the time expressed in minutes after the injection of test fluid,  $P = f(\sqrt{T})$ . For the initial intraarticular pressure in each experiment expressions were obtained by extrapolating to zero time.

The results obtained are given in table 7 p. 63 and figs. 11, 12, 13, 14. As the degree of joint flexion is varied the intraarticular hydrostatic pressure evidently adjusts itself at significantly different levels. Moreover, in animals with joints flexed 0° and 60° the initial pressures obtained by extrapolation to zero time apparently lie below the breaking point, and at a flexion of 90° the initial pressure significantly exceeds the breaking point. In animals with pre-exercised joints the initial pressure lies below the breaking point and is statistically the same as that found in normal rabbits with the same degree of joint flexion.

THE EFFECT OF PRESSURE ON FLUID FLOW THROUGH THE SYNOVIAL MEMBRANE IN RABBITS WITH PRE-EXERCISED JOINTS. The perfusion experiments gave the results shown in table 7 and figs. 15, 16.

It is obvious from the illustrations and the table that no breaking point existed in the majority of cases; at all pressures applied the flow was directly proportional to the pressure. In 5 of the 13 cases there was a breaking point, which indicates that the exercise had not affected the structure of the synovial membrane.

The following questions must be considered before the above results can be discussed and related to joint absorption under similar conditions.

1. Is the absorbent area in the joint cavity approximately constant irrespective of the flexion?
2. Is the above described disappearance of the breaking point somehow connected with a demonstrable edema in the synovial membrane 16—18 hours after the runs in the tread-mill?



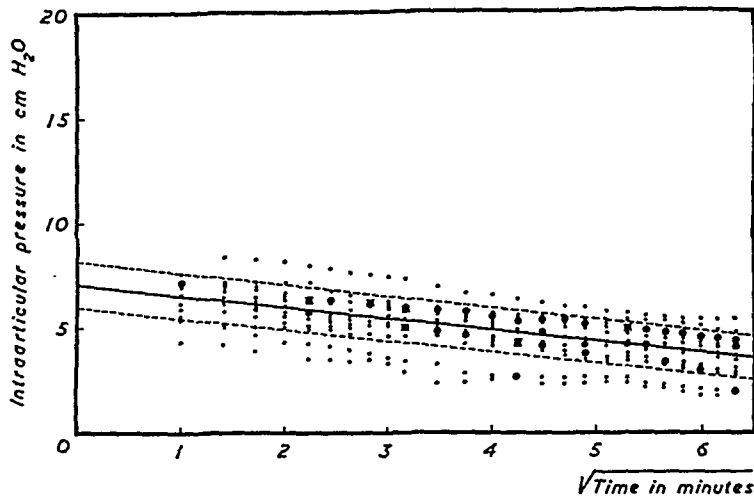


Fig. 11. Measurements of intraarticular hydrostatic pressure. Joint flexion  $0^\circ$ . 0.85 ml hemoglobin solution injected at  $0'$ . Full line; regression curve, dotted line; standard error of estimate. Different signs at the same point of the graph indicate coinciding values. 13 experiments. Test of linearity:  $P$  (probability that the assumption of linearity of regression is discredited)  $< 0.01$ . Average correlation coefficient calculated from the regression curve of each experiment and used as a measure of linearity  $= -0.979$ . Range of correlation coefficients for the 13 experiments  $0.996-0.745$ . For used methods of calculation of the test of linearity and average correlation coefficient see page 8.

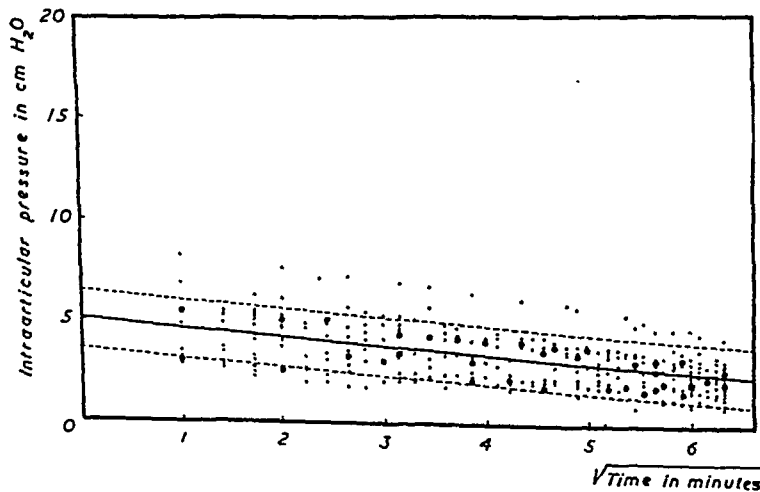


Fig. 12. Measurements of intraarticular hydrostatic pressure. Joint flexion  $60^\circ$ . 0.85 ml hemoglobin solution injected at  $0'$ . Full line; regression curve, dotted line; standard error of estimate. Different signs at the same point of the graph indicate coinciding values. 16 experiments. Test of linearity:  $P < 0.01$ . Average correlation coefficient  $= -0.966$ . Range of correlation coefficients  $= 0.995-0.643$ . (For definition of terms and symbols see the caption of fig. 11.)

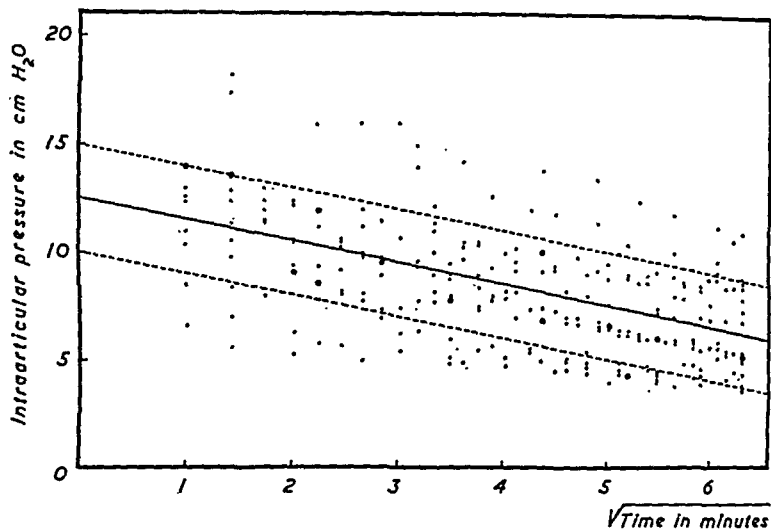


Fig. 13. Measurements of intraarticular hydrostatic pressure. Joint flexion  $90^\circ$ . 0.85 ml hemoglobin solution injected at 0'. Full line; regression curve, dotted line; standard error of estimate. Different signs at the same point of the graph indicate coinciding values. 15 experiments. Test of linearity:  $P < 0.01$ . Average correlation coefficient =  $-0.971$ . Range of correlation coefficients =  $0.992-0.916$ . (For definition of terms and symbols see the caption of fig. 11.)

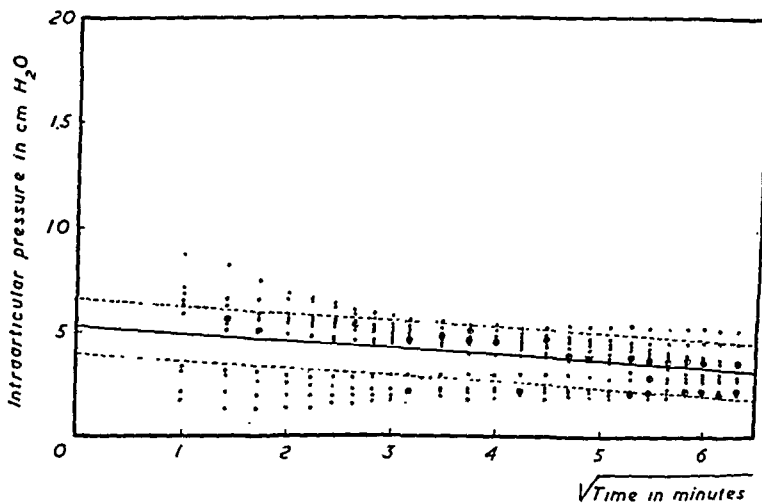


Fig. 14. Joint flexion  $60^\circ$ . Measurements of intraarticular hydrostatic pressure. Preexercised rabbits. 0.85 ml hemoglobin solution injected at 0'. Full line; regression curve, dotted line; standard error of estimate. Different signs at the same point of the graph indicate coinciding values. 12 experiments. Test of linearity:  $P < 0.01$ . Average correlation coefficient =  $-0.930$ . Range of correlation coefficients =  $0.983-0.333$ . (For definition of terms and symbols see the caption of fig. 11.)

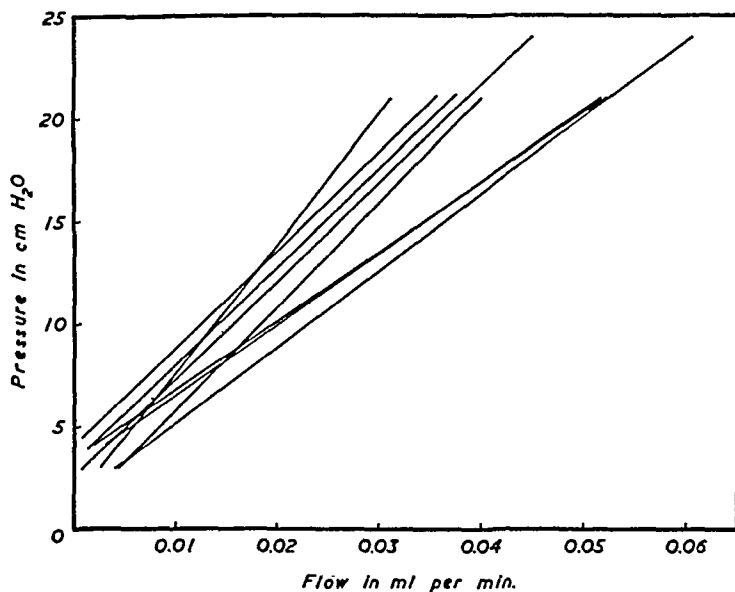


Fig. 15. Preexercised rabbits. No breaking point found in the experiments. Initial perfusion pressure about 3.0 cm H<sub>2</sub>O. Curves start at pressures where measurable inflow first could be registered and end at the highest used pressure. Regression curves calculated according to the method of least squares. Average correlation coefficient (see caption to fig. 11) = 0.991. Range of correlation coefficients = 0.996—0.981.

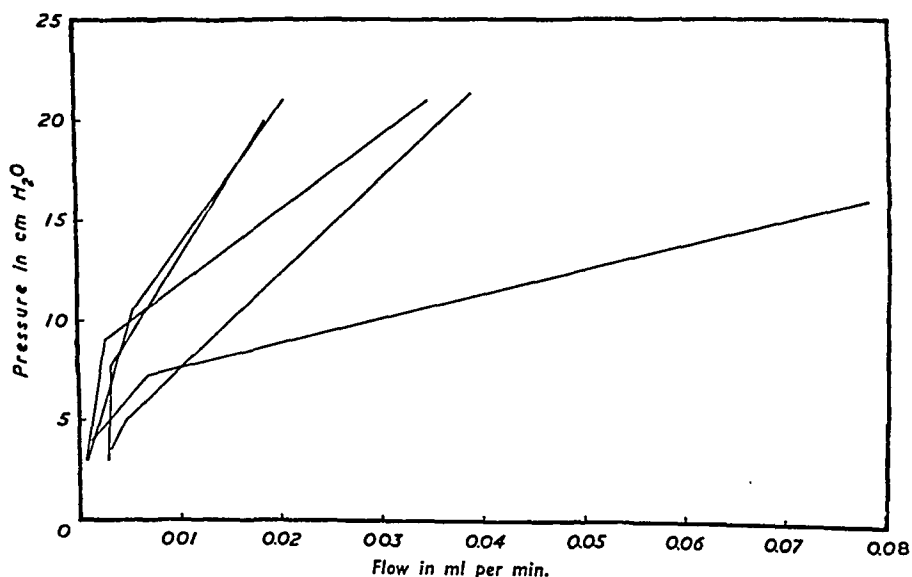


Fig. 16. Preexercised rabbits. Experiments giving a breaking point. Initial perfusion pressure about 3 cm H<sub>2</sub>O. Curves start at pressures where measurable inflow first could be registered and end at the highest used pressure. The two regression curves in each experiment calculated according to the method of least squares.

Ad. 1. By means of X-ray photos the areas of the joint cavities were studied at each degree of flexion. The amounts of contrast medium injected and the degree of flexion are given in the text to the figure (fig. 30 p. 104). Judging by the illustrations there evidently is no difference in the size of the absorbent area.

Ad. 2. 10 rabbits were exercised as described. After 16—18 hours the rabbits were urethane-anesthetized. After one hour's lapse they were killed by a rapid intravenous injection of some ml of the same anesthetic. The joint cavities were then opened medially by a longitudinal incision from the upper end of the tibia to the fornix of the suprapatellar recess. The patellar tendon and the quadriceps muscle having been cut transversally, a ligature was stitched through the free medial edge of the joint capsule in level with the patellar bone. Then the preparation was everted laterally and supported on a horizontal plate by applying tension to the ligature. Thereafter the internal surface of the joint capsule was inspected through a dissection microscope at a magnification of 10—100 diameters. This crude method gave no evidence of lesions or edema. The joint capsules in "treadmilled" rabbits did not pit on pressure, nor did free fluid ooze out through a puncture wound made with a very sharply ground dissection needle.

That a pronounced edema in the joint membrane actually causes pitting on pressure and exudation of fluid through a dissection needle puncture in the tissue was demonstrated in 3 cases where 0.5 mg histamine dihydrochloride in 0.5 ml Ringer had been injected intraarticularly 30 min. before the animals were killed.

*The conclusions drawn from these findings were that no pronounced edema existed in the synovial membrane; possibly there might have been a slight edema. The findings are dealt with more fully in the discussion of this chapter.*

## Part II

From the results arrived at in part I of this chapter the following facts are plain.

1. Different intraarticular hydrostatic pressures can be induced in joint cavities by using various degrees of flexion of the legs and the same amount of injected test fluid in the different animal groups.

2. The initial intraarticular pressure can be adjusted to a level either above or below the breaking point of the synovial membrane.

3. In the majority of cases the breaking point of the synovial membrane disappears by pre-exercising the joints. This procedure does not significantly change the intraarticular hydrostatic pressure level as compared with animals not so treated, but having the same degree of joint flexion and the same amount of test fluid injected.

Thus, provided a suitable method of measuring is developed, it would be possible to determine the influence on joint absorption of intraarticular pressure and a vanished breaking point.

#### Method of Determining the Absorption of Colloid and Fluid from Joints

**TEST FLUID.** A 5.5 g per cent solution of human hemoglobin was employed. The rabbits were anesthetized as before. The insertion of the needles into the joint cavities is fully described on p. 17.

**DEGREE OF JOINT FLEXION.** When nothing is said to the contrary the joints were flexed  $60^\circ$  from the extended position. The degree of abduction may be seen in fig. 29 b, p. 103.

**GENERAL PERFORMANCE OF THE TEST.** The absorption test began by the insertion of the needles 10 minutes after the end of the urethane injection. 0.85 ml of the hemoglobin solution, warmed to  $38^\circ\text{C}$ ., was injected with a calibrated hypodermic syringe into the joint cavities, the right one 5 minutes before the left. The time lag between the insertion of the needle and the injection of hemoglobin was the same in both joints.

After the injections the needle sockets were closed with exactly fitting stoppers. At the end of the set absorption time 0.05 ml of the intraarticular test fluid was withdrawn as follows with the aid of a small calibrated all-glass syringe. About 0.2 ml was 5 times aspirated and reinjected in order to thoroughly mix the contents of the joint cavity. About the same amount of test fluid was then removed in the syringe and 0.05 ml of it was used to determine the concentration of hemoglobin. The remainder of the liquid in the syringe was reinjected into the joint cavity. Immediately afterwards the joint cavity was washed out by 10 consecutive injections and aspirations with a tuberculin syringe holding an exact amount of fluid, usually 1.04 ml.

A sample for hemoglobin determination was taken from the washing fluid.

The volume of the joint cavity is easy to calculate from the hemoglobin concentrations before and after washing. The figure thus obtained must, however, be corrected for the sample withdrawn for hemoglobin determination and the residual volumes in the all-glass syringes. This corrected figure represents the volume of the intra-articular fluid at the time of taking the first concentration sample.

The amount of hemoglobin absorbed from the joint cavity during the test period may now be calculated from the volume and the concentration thus found. The volume found at the end of such a period subtracted from the volume of fluid injected at the beginning of the experiment is a measure of the net fluid absorption during the test period.

The following points must be taken into consideration before these statements can be taken at a face value.

*A. Is the washing of the joint cavity complete, or, in other words, is the obtained residual volume the true one, and does, consequently, the calculated amount of test fluid at the end of the test period represent the amount remaining unabsorbed in the joint?*

In order to determine whether the recovery is complete of an intra-articularly injected test substance or fluid an indicator is required which remains unabsorbed during the interval between its injection and the final washing, it must also have about the same specific gravity as the washing fluid, and it must not be precipitable by the synovial fluid. An indicator satisfying these criteria is prepared as follows.

Three times washed human blood corpuscles were agglutinated with complement-inactivated human serum from a person belonging to blood group 0.<sup>1</sup> The amount of serum was about double that of washed blood corpuscles. Agglutination was done in a small centrifuge tube holding about 10 ml and the suspension was allowed to stand overnight at 4° C. Immediately before use the tube was shaken until its contents were macroscopically homogeneous. Then the tube was placed in a rotary mixer similar to the type described by ENGHOFF (1937), running at 30 revolutions per minute for 5 minutes, which

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<sup>1</sup> All the samples of blood corpuscles and serum were taken from the same donor of serum and corpuscles, respectively. The agglutinating titre of the serum was  $1/512$  as determined by the method described by WIENER (1946, p. 17), 0.5 ml of a 2.5 per cent corpuscle suspension was agglutinated with 0.5 ml of serum and twofold dilutions thereof.

proved to be enough; 5 and 10 minute runs giving identical hemoglobin concentrations when specimens were taken at the top and the bottom of the tube.

After mixing for 5 minutes microscopical inspection of the agglutinated red corpuscle aggregates through a micrometer eyepiece revealed that most of the aggregates were of the magnitude 30—50 microns.

Each tube of agglutinated red blood corpuscles was used in one experiment only since it turned out that some of the corpuscles were hemolyzed if they were mixed for more than 10 minutes.

Control Tests. 0.85 ml of the corpuscle suspension was injected with the calibrated syringe mentioned above.

The syringes used in taking the concentration samples were the ones mentioned under "General performance of the test".

The following types of experiments were carried out.

1. 0.85 ml of corpuscle suspension was injected. After 30 seconds one of the all-glass syringes was fitted in the socket of the intraarticularly placed needle as described above. The intraarticular contents were withdrawn and reinjected 5 times and then 0.05 ml of the joint contents were taken as described in the foregoing concerning determinations of the hemoglobin concentration. Having reinjected the fluid remaining in the syringe after sampling, the joint cavity was washed as described above with 1.04 of physiological saline. 0.1 ml of the washing solution was taken for determination of the hemoglobin concentration.

2. The same as the above experiment except that the amount of washing fluid was 0.50 ml.

3. In order to rule out the possibility that the values obtained from the above two types of experiments had been affected by the colloid osmotic pressure of the serum suspension of blood corpuscles the latter were prepared as follows.

Washed blood corpuscles were agglutinated with about 10 volumes of the agglutinating serum. Having stood for about 1 hour at room temperature the mixture was centrifuged, the supernatant drained off and physiological saline added in an amount sufficient to bring the volume of the suspension to about twice the volume of the corpuscles. The agglutinate was disintegrated by agitation and the macroscopically homogenous suspension put into centrifuge tubes and allowed to settle overnight at 4° C. This suspension was then treated like

TABLE 2.

Type and volume of injected indicator suspension. Volume of washing fluid (0.9 per cent Na Cl)	Recovered amount of indicator in per cent of injected
Agglutinated blood corpuscles in serum <sup>1</sup> Volume injected = 0.85 ml Volume of washing fluid = 1.04 ml Number of joint = 18	$m = 100$ $e = \pm 2.2$
Agglutinated blood corpuscles in serum <sup>1</sup> Volume injected = 0.85 ml Volume of washing fluid = 0.50 ml Number of joints = 14	$m = 97$ $e = \pm 4.0$
Agglutinated blood corpuscles in saline <sup>1</sup> Volume injected = 0.85 ml Volume of washing fluid = 1.04 ml Number of joints = 11	$m = 100$ $e = \pm 2.2$

<sup>1</sup> Concentrations of hemoglobin in the used corpuscle suspensions 6—9 g per cent.

Signs:  $m$  = arithmetic mean,

$e$  = standard error of the mean.

the one containing serum: with respect to hemolysis and aggregate magnitude it exhibited the same properties as the latter. Tests were then carried out exactly as under 1.

*These tests demonstrated that the washing of the joint cavity seems to be complete, that the obtained residual volume seems to be the true one, and that consequently the calculated amount of test fluid at the end of the test period seems to represent the amount remaining unabsorbed in the joint. (Table 2.)*

**B.** *Are the values obtained uninfluenced by the intermittent variations in pressure which are a result of the washing?*

Even if in the above control experiments the indicator is totally recovered, the present author contends that this possibly might be caused by some systematic error of method.

During the washing with saline the intraarticular pressure changes intermittently and must on the whole be far higher than the pressure caused by the injection of test fluid only. This might cause some of the test fluid to be forced through the synovial membrane while simultaneously the indicator, agglutinated red blood corpuscles, remains in the joint cavity. Such being the case the concentration of indicator in the washing fluid would be higher than would have been the case had there been no leakage of fluid during the washing



procedure. This possibility implies that the calculated amount of indicator recovered can amount to 100 per cent despite the existence of a fraction of the intraarticularly injected indicator which is inaccessible to the washing fluid. In spite of the two different volumes of washing fluid that have been injected the three kinds of tests listed do not exclude the possibility of leakage. This reasoning is justified by the fact that a smaller volume of washing fluid must necessarily give a lower intraarticular pressure and less leakage, if any. By chance smaller leakage might in the latter case give the same degree of recovery as in the experiments where larger volumes were used. The possibility exists on the other hand that the accessible volume of injected indicator is smaller when a smaller washing volume is used. Owing to the lower pressure during the washing in the latter case the recesses in the intraarticular cavity might be less completely unfolded. The possible influence of these systematic errors are best shown by the following calculations.

Suppose that the volume and concentration of the injected indicator do not change in the joint before the washing procedure has begun.

Volume of injected indicator	= $V$ ml
Concentration of indicator in $V$	= $C_p$
Washing volumes	= 1.04 and 0.50 ml
Fractions of the injected volumes accessible to washing, the volumes being 1.04 and 0.50, respectively	= $\beta V$ and $\beta_1 V$
Washing leakage (1.04 ml)	= $\alpha$ ml
Washing leakage (0.50 ml)	= $\alpha_1$ ml
100 per cent recovery of indicator in both cases	= $V \cdot C_p$

Hence the indicator concentration in the washing fluid must be:

$$\begin{aligned}
 1.04 \text{ ml washing volume} &= \frac{C_p \cdot \beta V}{1.04 + \beta V - \alpha} \\
 0.50 \text{ ml washing volume} &= \frac{C_p \cdot \beta_1 V}{0.50 + \beta_1 V - \alpha_1}
 \end{aligned}$$

Since we have complete recovery in both cases the following identity must be valid:

$$\frac{0.5 + V}{1.04 + V} = \frac{0.5 + \beta_1 V - \alpha_1}{1.04 + \beta V - \alpha} \times \frac{\beta V}{\beta_1 V}.$$

If we introduce numerical values into the formulae it is obvious that identity can only exist if  $\alpha_1 < \alpha$  when  $\beta V = \beta_1 V$ .

If the volume accessible to the washing is smaller in the case when 0.50 ml

washing fluid was used, then complete recovery in both cases also here depends on  $\alpha_1$  and  $\alpha$ . However, a small leakage, which is equal in both cases, may be compensated by a smaller volume accessible to washing in the case when a smaller washing volume was used. Hence these control tests prove nothing as to the general validity of the method, at any rate whenever  $\beta V$  and  $\alpha$  are not determined.

The following experiments were carried out to ascertain whether any fluid leaked out during the washing and whether only a fraction of the intraarticularly injected fluid was accessible to washing.

0.85 ml of an agglutinated corpuscle suspension in serum was injected intraarticularly as described before. The washing was then performed with a suspension of agglutinated red blood corpuscles in saline. The volume of the washing fluid was 1.04 ml.

Assuming that no aggregates are absorbed from the joint cavity during the washing — which is improbable owing to their average diameter — the intraarticular volume occupied by the indicator calculated from the sample taken at 30 seconds + 1.04 ml (the volume of washing fluid used) ought to be equal to the volume of the articular cavity and syringe + the residual volume in the syringe used in taking the 30 seconds sample + the volume of this sample when the volume of the articular cavity and the syringe are computed from the hemoglobin concentration after washing in the washing fluid.

The above is best illustrated by the following:

Volume of indicator injected	= 0.85 ml
Hemoglobin concentration in indicator	= $C_p$
Volume of washing fluid	= 1.04 ml
Hemoglobin concentration in washing fluid	= $C_w$
Concentration of hemoglobin in sample taken 30 seconds after the injection of indicator	= $C_s$
Hemoglobin concentration in sample of the washing fluid after washing	= $C_{ws}$
Residual volume in syringe used to take sample for determination of $C_s$ + volume of sample	= 0.073 ml
Intraarticular volume 30 seconds after injection of indicator	= $\frac{C_p}{C_s} \cdot 0.85 = V_1$
Capacity of articular cavity + syringe, calculated from the hemoglobin concentration in the washing solution after washing	

$$= V_{11} = \frac{C_p \cdot 0.85 + 1.04 \cdot C_w - 0.073 \cdot C_s}{C_{ws}}.$$

Under ideal conditions (complete washing, no leakage)

$$V_{11} + 0.073 = V_1 + 1.04.$$

TABLE 3.

Type and volume of injected indicator suspension. Type and volume of washing fluid	$\frac{\sum (V_{11} + 0.073 - V_1 - 1.04)}{17} = D_m \text{ ml}$
Indicator: Agglutinated blood corpuscles in serum Volume injected = 0.85 ml Concentration of hemoglobin in indicator suspension = $C_p$  Washing fluid: Agglutinated blood corpuscles in saline Volume of washing fluid = 1.04 ml Concentration of hemoglobin in washing fluid = $C_w$  Number of experiments = 17  In listed experiments $C_w \sim 2 C_p$ Range of $C_p$ : 3.5-9.5 g per cent	$D_m = 0.02 \text{ ml}$ $c = \pm 0.02 \text{ ml}$

Signs:  $D_m$  = arithmetic mean of differences  $D$ .

$c$  = standard error of  $D_m$ .

For explanation of  $V_1$ ,  $V_{11}$ , and constants 0.073 and 1.04 see text.

Before more fully discussing these controls the possible influence of fluid leakage and the possibility that only a fraction of the intraarticular fluid is accessible for washing must be considered. (The possibility that corpuscle aggregates are forced out of the joint cavity during the washing is improbable owing to their average diameter.)

Suppose that  $C_p = C_s$

Then  $V_1 = 0.85 \text{ ml} = V$

Washing leakage  $= a \text{ ml}$

Volume of indicator accessible for washing  $= \beta V$

Since in this theoretical case we can neglect the quantity of hemoglobin withdrawn in the 30 second sample and the residual volume in the syringe used for the sampling we get:

$$C_{ws} = \frac{\beta V \cdot C_p + 1.04 C_w}{\beta V + 1.04 - a}$$

Suppose that the controls give a calculated complete recovery of volume then we get:

$$(V + 1.04) \frac{\beta V \cdot C_p + 1.04 C_w}{\beta V + 1.04 - a} = V \cdot C_p + 1.04 C_w$$

This expression may be transformed into:

$$\frac{C_w}{C_p} (1 - \beta) + \beta - 1 + \frac{a}{1.04} + \frac{a}{V} \frac{C_w}{C_p} = 0.$$

If  $C_w > C_p$  and the experiments result in  $V_{11} + 0.073 = V_1 + 1.04$  leakage during the washing and/or only a fraction of the injected indicator being accessible to the washing fluid can then be neglected as falling within the error of the method.

By the controls just described it was proved *that the obtained values are uninfluenced by measurable leakage and incomplete washing procedure.* Table 3.

**C.** *Is the method reliable when the resistance to flow of the synovial membrane is lowered, i. e. when its permeability is very great?*

The following artefacts may exist with reference to the type of experiments described under A and B.

It may occur that the red blood corpuscle aggregates block the pores in the synovial membrane, thereby preventing an outward passage of fluid, which would have occurred attending the intermittent increase in pressure caused by the washing of the articular cavity, had not the washing fluid been a corpuscular suspension. In biological systems such decreased permeability caused by particulate matter has been found by DANIELLI (1940) who used thrombocytes as particles and by CHAMBERS and ZWEIFACH (1940) who suspended red cell ghosts and carbon particles in the perfusion fluid. These authors attributed the observed decrease in capillary permeability to a blocking of the intercellular pores in their perfused preparations. Moreover, complete recovery of the indicator in the above control experiments by no means implies that the method of determining the absorption of colloid and water from joints is valid when the resistance to flow of the joint membrane is lessened, i. e. when the breaking point is absent.

The following experiments were performed in order to demonstrate the absence of these artefacts.

A slight edema in the synovial membrane should induce a lower resistance to flow in the synovial membrane. When absorption tests with two different washing fluids — isotonic saline and suspensions of agglutinated red blood corpuscles — are carried out on such pre-treated joints, one might expect that a possible blocking effect of the corpuscle suspension and/or a leakage of fluid during washing with saline or corpuscle suspension could, provided that the latter fluids had suitable hemoglobin concentrations, be demonstrated as numerical differences between the obtained absorption figures.

Since it has been shown in the above that under certain circumstances it can be determined that all the injected indicator is accessible for washing, the following reasoning holds provided that such is actually the case.

Volume of washing fluid	= 1.04 ml
Hemoglobin concentration in washing fluid with corpuscle aggregates	= $C_w$
Hemoglobin concentration in washing fluid after washing with corpuscle suspension in saline	= $C_{wsc}$
Volume of intraarticular contents 40 minutes after injection of hemoglobin solution into the joints	= $V_{40}$ ml
Hemoglobin concentration in $V_{40}$ determined from sample taken immediately before washing	= $C_p$
Volume of above sample + residual volume in syringe used in sampling	= 0.073 ml
Washing leakage	= $\alpha$ ml
Non-corpuscular hemoglobin concentration in $\alpha$	= $C_\alpha$

It can be shown that

$$V_{40} = \frac{\frac{C_w}{C_{wsc}} - 1}{1 - \frac{C_p}{C_{wsc}}} \cdot 1.04 + 0.073.$$

If leakage takes place during the washing the calculated volume is not  $V_{40} - 0.073$  ml but another  $V$ .  $V_{40} - 0.073 = V_n$

Then we get

$$\frac{V}{1.04} = \frac{V_n + \frac{\alpha(C_w - C_\alpha)}{C_p - C_w}}{1.04 + \frac{\alpha(C_\alpha - C_p)}{C_p - C_w}}.$$

1. Suppose that

$$\begin{aligned} C_{wsc} &> C_p \\ C_w &> C_{wsc} \end{aligned}$$

Since it was assumed that all the intraarticular fluid was accessible for washing the non-corpuscular hemoglobin in the joint of concentration  $C_p$  is certainly lessened, even after the first injection of washing fluid.

Hence  $C_\alpha < C_p$ .

Then the obtained value for  $V$  must always be smaller than the real one

2. Suppose that

$$\begin{aligned} C_{wsc} &< C_p \\ C_w &< C_{wsc} \end{aligned}$$

Then  $V + 0.073 > V_{40}$  when  $C_w > C_\alpha$ .

It cannot be proved that such is actually the case after, for example, the first injection and aspiration of washing fluid. However, since the washing procedure takes about 30 seconds the leakage taking place when  $C_a > C_w$  cannot in the opinion of the present author have any measurable influence. This is best illustrated by the following.

Assume that — as in the actual experiments —  $C_w \sim \frac{1}{2} C_p$  and  $V_{40} \sim 0.50$  ml. Then, in order to give values of  $V$  higher than  $V_{40}$ , only 0.50 ml of the injected washing fluid need have been mixed with  $V_{40}$  to make  $C_w > C_a$ . Undoubtedly, such incomplete mixing is possible at the beginning of the washing procedure only, perhaps only during the first injection of washing fluid.

If the values obtained for  $V_{40}$  are statistically identical — when  $C_w > C_p$  and  $C_w < C_p$  — and also identical to the values obtained in similar experiments where physiological saline is used as washing fluid, the conclusions must be drawn that

- a) Irrespective of the washing fluid there is no leakage during the washing, or*
- b) the tissue pores become completely blocked when corpuscular suspensions are used, and*
- c) the leakage, if any, taking place when saline is used necessitates that  $C_a =$  the hemoglobin concentration after washing in the washing fluid.*

*Thus, it may be stated that no systematic errors are inherent in the method as long as the above requirements are fulfilled.*

**Control Tests.** The animals were anesthetized and needles inserted into the joint cavities as before. 0.5 ml of the supernatant of a centrifuged, 24-hours old culture of the staphylococcus strain (described previously) was after dilution injected intraarticularly (1 part of culture to 5 of saline). The right and the left knee joints were alternately injected first, the interval between the injections being 5 minutes. The time lag between the insertion of the needles and the injection of the diluted supernatant was the same in both joints. 30 minutes after the injection of irritant the fluid in each joint was aspirated with a syringe and 0.85 ml of hemoglobin solution was injected intraarticularly. 40 minutes after the hemoglobin instillation a sample was taken for determination of the intraarticular hemoglobin concentration. Then the washing procedure was carried out as follows.

In alternate rabbits the first injected joint was washed with 1.04 ml

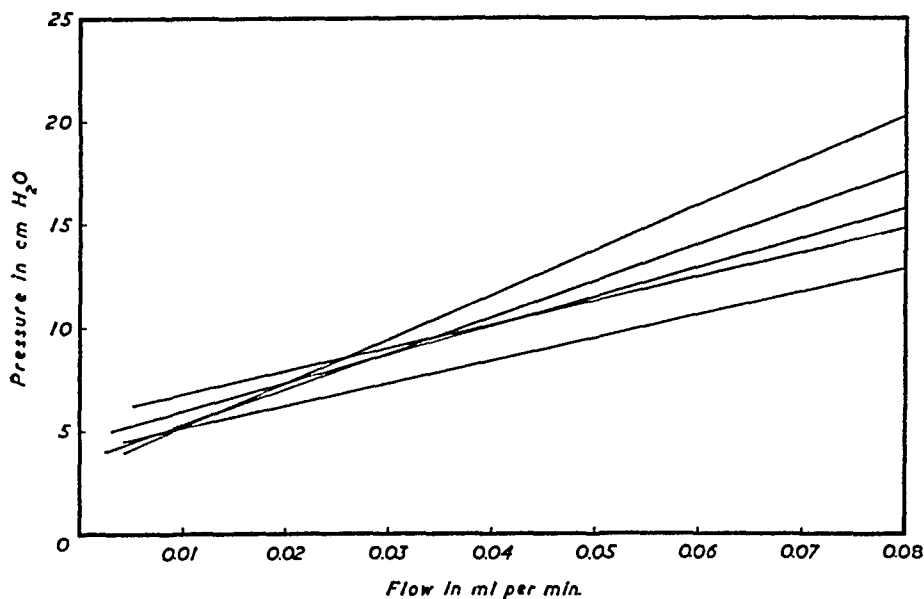


Fig. 17. Rabbits injected intraarticularly with 0.5 ml of a diluted staphylococcus culture filtrate 30 minutes before the perfusion experiments began. Curves start at pressures where measurable inflow first could be registered and end at the highest used pressure. Average correlation coefficient (see text to fig. 11) = 0.989.

of physiological saline; the second joint with a suspension of agglutinated red blood corpuscles, 1.04 ml in volume. In the other animals washing was performed the other way about. The calculated amounts of hemoglobin and the net. amount of fluid absorbed were then registered and classed according to the type of washing fluid used.

In order to prove that the resistance to flow of the synovial membrane was altered by the bacterial filtrate 5 joints were subjected to the test described in chapter III. In these joints the filtrate was injected as above and 30 minutes after the injection the contents of the joint was withdrawn, 0.5 ml Ringer solution injected and perfusion experiments performed as described elsewhere. (Fig. 17.)

The results of the control experiments to check the validity of the method of determining the absorption of fluid and colloid from joints are shown in table 4. From the table it is evident that the method is satisfactory and that no measurable systematic errors exist.

Hence, it is true that *the method is reliable when the resistance to flow of the synovial membrane is lowered, i. e. when its permeability is very great.*

TABLE 4.

At 0' injection of the diluted culture-supernatant in the joint first to be tested, at 5', in the other. Absorption experiments start 30' after injection of irritant. 0.85 ml 5.5 g per cent hemoglobin solution in each joint. Test period 40'. Degree of joint flexion, 60°. 20 animals used	Absorbed hemoglobin in percent of injected	Volume in ml of intraarticular fluid at end of test period	Concentration in g per cent of hemoglobin at end of test period
5 right, 5 left knee joints. Concentration of hemoglobin in the corpuscle suspension used as washing fluid: $C_w \sim 2 C_p$	$m = 62.6$ $e = \pm 2.0$	$m = 0.496$ $e = \pm 0.032$	$m = 3.59$ $e = \pm 0.15$
5 right, 5 left knee joints. Concentration of hemoglobin in the corpuscle suspension used as washing fluid: $C_w \sim \frac{1}{2} C_p$	$m = 62.4$ $e = \pm 2.3$	$m = 0.508$ $e = \pm 0.033$	$m = 3.49$ $e = \pm 0.13$
10 right, 10 left knee joints. Washing fluid 0.9 per cent saline. One joint in each animal used with this washing fluid	$m = 61.8$ $e = \pm 1.7$	$m = 0.536$ $e = \pm 0.025$	$m = 3.32$ $e = \pm 0.06$

Signs:  $m$  = arithmetic mean

$e$  = standard error of the mean

For explanation of  $C_w$  and  $C_p$  see text.

**D. Can the occurrence of intraarticular hemorrhage be a source of error in the method?**

Significant intraarticular hemorrhage as a cause of error in the experiments was avoided by excluding all animals with a red blood cell count above 8,000 per cu. mm in the washing fluid after washing. In the series with hemolytic solutions injected into the joints the counting of corpuscles and/or ghosts took place in a phase-contrast microscope, which made the ghosts visible. A regular type of counting chamber was employed.

Such controls are impossible in experiments where corpuscles are used either in the washing fluid or as indicator. However, experience gained from about 2,000 absorption tests reveals that intraarticular hemorrhage in an amount sufficient to invalidate the experiment occurs in 2—3 per cent of the cases. Therefore, the author considered it unnecessary to discuss intraarticular bleeding as a source of error in the experiments described under A, B; and C.

Hence, *intraarticular hemorrhage is not a source of error in the method.*



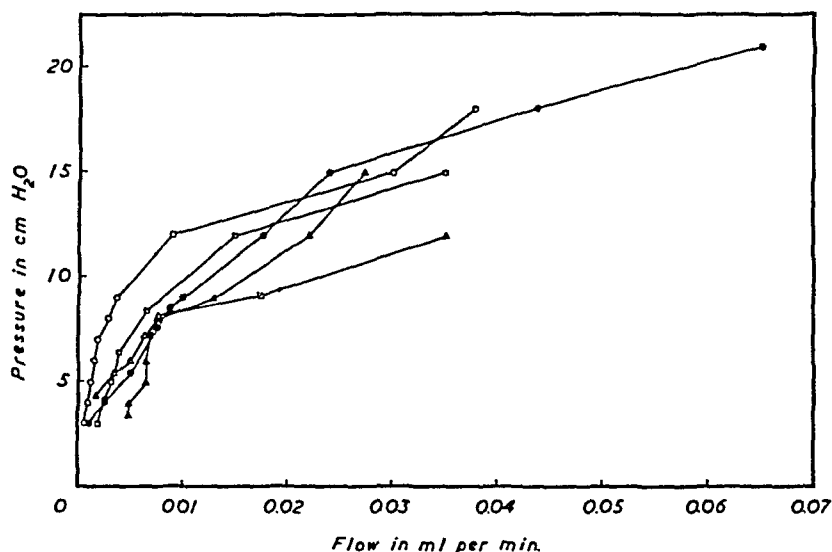


Fig. 18. Perfusion experiments on rabbits injected intraarticularly with 0.85 ml hemoglobin solution 40 minutes before the perfusion experiments started. Signs indicate perfusion pressure levels and corresponding flow rates.

*E. Does the hemoglobin solution influence the resistance to flow of the synovial membrane?*

Hemoglobin solution (0.85 ml) was injected into the knee joints of 5 rabbits in the manner described on page 33. After 40 minutes the needle was connected to the perfusion apparatus described in chapter III. No Ringer solution was injected into the joint cavity before the perfusion experiment was started. Otherwise the experiments were carried out exactly as described, using perfusion periods at each applied pressure of 4—6 minutes.

The results of the experiments to test the action of the hemoglobin solution on the resistance to flow of the synovial membrane are given in fig. 18. Evidently a breaking point exists in all these cases. This agrees with the findings of Mc MASTER (1941 c) who used homologous serum as test fluid. Therefore the hemoglobin solution employed has no effect on the course of the pressure/perfusion curve which is the same as that in experiments where Ringer solution was used.

Hereby it appears that *the hemoglobin solution used does not cause any structural changes as measured here in the synovial membrane.*

## Part III

## The Absorption of Colloid and Fluid from Normal Joints and the Effect on this Phenomenon of Intraarticular Pressure

THE ABSORPTION OF COLLOID AND FLUID FROM JOINTS WHERE THE BREAKING POINT NO LONGER EXISTS OWING TO PREVIOUS EXERCISE OF THE ANIMALS.

The absorption of hemoglobin and fluid was calculated for each knee separately and in the subsequent statistical calculations the mean of the two joints was treated as one observation. The experiments were arranged as follows.

1. 60° joint flexion. These experiments were arranged with a view to investigate systematic variations in the results. The following variations are conceivable.

a. Since animals classed only by body weight might not be of the same age during different seasons and it is known that in rabbits the permeability of at any rate dermal connective tissue is greater in young (25—60 days old) than in adult animals (DURAN-REYNALS, unpublished experiments, quoted from DURAN-REYNALS (1942)) and that in rabbits the adult type of permeability of dermal connective tissue is manifested in animals older than three months (LURIE and ZAPPASODI, 1939), one series of animals was studied during the months December—February and another during August.

b. Since the pH of the test solutions remains constant and the hemoglobin/methemoglobin ratio must change with the storage time of the test solution this change might affect the absorption value obtained. The colloid osmotic pressure is said to be the same in solutions of hemoglobin and methemoglobin (ADAIR *loc. cit.*).

In order to investigate if the storage time of the hemoglobin solution in any way affects the absorption values, a series of animals was tested with three fresh solutions of hemoglobin and the results compared with the results obtained in the series described above under 1 a where three different hemoglobin solutions also were used in each series but where the storage time amounted to 4—14 days. See table 5 for results.

Oncotic pressure during the test period of the injected hemoglobin solution. Since the oncotic pressure of the injected hemoglobin solution must affect fluid exchange through the synovial

TABLE 5.

Intraarticular injection of 0.85 ml of hemoglobin solution. Mean values from right and left knee joint in each animal=one experiment. Duration of experiment = 40 minutes. Degree of joint flexion: 60°	Animals used in December-February. Storage time of the three different hemoglobin solutions: 4-14 days. 16 experiments	Animals used in August. Storage time of the three different hemoglobin solutions: 4-14 days. 15 experiments	Animals used in August. Storage time of the three different hemoglobin solutions: 1-2 days. 15 experiments
The amount of hemoglobin absorbed in per cent of the amount injected	$m = 50.3$ $e = \pm 1.7$	$m = 52.1$ $e = \pm 0.8$	$m = 51.8$ $e = \pm 1.0$
The intraarticular fluid volume in ml at the end of the test period	$m = 0.617$ $e = \pm 0.020$	$m = 0.592$ $e = \pm 0.013$	$m = 0.595$ $e = \pm 0.012$
The concentration of hemoglobin in g per cent in the intraarticular fluid at the end of the test period	$m = 3.77$ $e = \pm 0.07$	$m = 3.80$ $e = \pm 0.05$	$m = 3.79$ $e = \pm 0.05$

Signs:  $m$  = arithmetic mean  
 $e$  = standard error of the mean.

membrane during the test period, the values of this pressure were calculated from experiments carried out as follows. The hemoglobin solutions having been injected, samples were taken at 0, 5, and 20, minutes for determination of the hemoglobin concentration. This was done in the manner given on p. 33. Washing was then performed after the samples taken at 5 and 20 minutes and the values calculated in the usual manner for the absorption of hemoglobin and fluid (table 6).

Then the oncotic pressures of the hemoglobin solution at 0, 5, 20 and 40 minutes after the injection of the test fluid were calculated by the formula given by ADAIR (1928) and corrected for temperature according to WELLS *et al.* (1935).

It turned out that the oncotic pressure exerted in the joint by the hemoglobin solution only could be expressed as a function of the square root of the time in minutes after the injection of the hemoglobin into the joint cavity. (Fig. 19.)

2. 0° Joint Flexion. These experiments were performed exactly as when the joint flexion was 60°. One joint in each animal was flexed 60° after taking the concentration sample before washing, the washing

TABLE 6.

Mean value from both joints in each animal = one experiment. Degree of joint flexion: 60°	Time in minutes after intraarticular injection of 0.85 ml hemoglobin solution. For values 40 minutes after injection see table 5 (animals tested in December—February)		
	0	5	20
The amount of hemoglobin absorbed in per cent of injected		$m = 33.2$ $e = \pm 0.8$ $n = 17$	$m = 37.6$ $e = \pm 1.4$ $n = 16$
The intraarticular fluid volume in ml at the end of the test period		$m = 0.690$ $e = \pm 0.011$ $n = 17$	$m = 0.693$ $e = \pm 0.017$ $n = 16$
The concentration of hemoglobin in g per cent in the intraarticular fluid at the end of the test period	$m = 5.26$ $e = \pm 0.04$ $n = 20$	$m = 4.54$ $e = \pm 0.05$ $n = 17$	$m = 4.23$ $e = \pm 0.07$ $n = 16$

Signs:  $m$  = arithmetic mean  
 $e$  = standard error of the mean  
 $n$  = number of experiments.

was then carried out in this position. Since joints washed at 0° and 60° flexion exhibited no difference in absorption values, the means from each rabbit were calculated as above.

3. 90° Joint Flexion. These experiments were carried out as under 2, i. e. one joint was washed at 60° flexion, the other at 90°. Nor in this case was there any difference; the mean values from each rabbit were therefore calculated as above.

4. 60° Joint Flexion in Rabbits Preexercised in a Treadmill 16—18 Hours before the Absorption Tests were Performed. The manner of exercising the rabbits is described on page 26. The absorption values and the hemoglobin concentrations at the end of the test period were calculated as described above for each rabbit.

The results of the experiments described in part III of this chapter appear in tables 5, 6, 7 (p. 63) and fig. 19. Clearly, no seasonal variations in the absorption figures or changed absorptive data caused by different storage times of the hemoglobin solution are measured. Furthermore, it is obvious that the intraarticular hydrostatic pressure apparently has no influence on the absorption of colloid and fluid or on the concentration of intraarticular hemoglobin before the

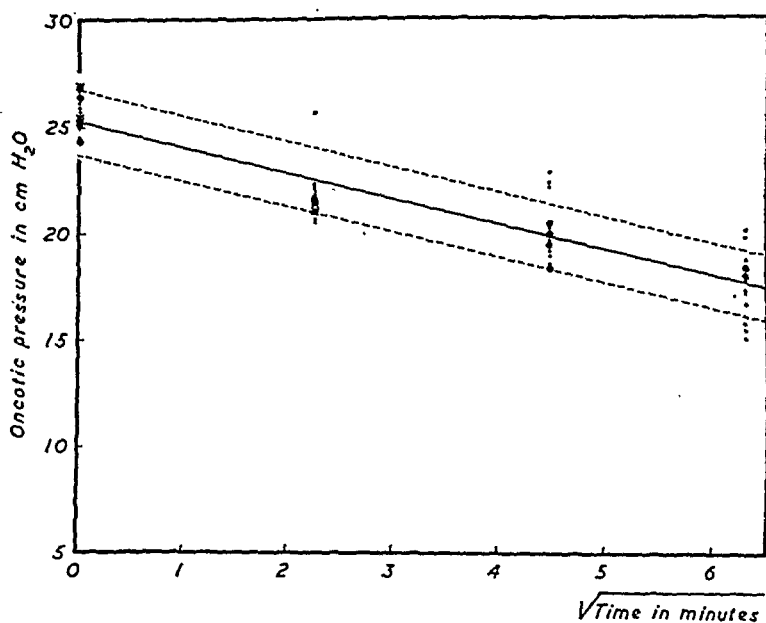


Fig. 19. Oncotic pressure exerted in the joint cavities by 0.85 ml 5.5 g per cent hemoglobin solution injected at 0'. Pressures are calculated from determinations of hemoglobin concentrations. Full line; regression curve, dotted line; standard error of estimate. Different signs in the same point of the graph indicate coinciding values.

washing as long as the initial hydrostatic pressure lies below the breaking point. When the initial hydrostatic pressure is above the breaking point the absorption of both colloid and fluid is increased and the hemoglobin concentration in the intraarticular fluid is higher than in the "normal cases". The same data, however, also applies to preexercised rabbits in the majority of which the breaking point is absent. (see p. 28).

## Part IV

### Discussion

#### Earlier Literature Dealing with Joint Absorption

Except for the paper of EDLUND and LINDERHOLM (1947) the literature seems to contain no papers on joint absorption where the amount of solute and solvent absorbed from the joint cavity has been measured directly, i. e. as differences between the amount of solute in the injected volume and that remaining in the joint cavity after a certain test period. BYWATERS *et al.* (unpublished experiments, quoted

from BAUER *et al.* 1940) claimed to have been able to determine the amount of phenolsulphonephthalein remaining in the knee joints of normal dogs one hour after injection, but the results have apparently never been published.

BAUER *et al.* (1933) found egg albumin and horse serum albumin in the thoracic duct lymph 30 minutes after intraarticular injections into the knee joints of dogs provided the legs were passively exercised or massaged. None of these proteins were serologically detectable in the blood stream when all communications between the lymphatic and vascular systems were tied off. Horse serum globulin was removed from the joint cavities with difficulty, if at all, when the joints were exercised. They concluded that the size of a molecule that can be readily removed from the joint cavity in dogs lies between the molecular weight of horse serum globulin and horse serum albumin. Their experiments do not permit of any conclusions as to whether the globulin molecules are withheld in the joint cavity or cannot permeate the endothelium of the lymphatic capillaries or if they are filtered off in the lymph nodules which the lymph from the joint cavity must pass before it reaches the thoracic duct. From experiments in which solutions containing mecholyl were intraarticularly injected RHINELANDER *et al.* (1939) concluded that mecholyl was readily absorbed from the joint cavity, and that this absorption was increased by passive exercise of the legs, as well as from joint cavities where mild and severe acute inflammations had been induced. Since they were able to counteract the fall in blood pressure caused by the intraarticular injection of mecholyl by intraarticular injections of adrenalin — which presumably constricted the subsynovial capillaries — and because the effect on blood pressure was evident within 30—60 seconds after the injection, they concluded that easily diffusible substances are removed from joints primarily via the blood capillaries.

The earlier literature on the subject of joint absorption in which the results were obtained by anatomical studies has been mentioned in chapter I of this paper. The consensus of opinion expressed in these investigations was that high molecular substances were conducted away from the joint cavity by the lymphatics, substances of small molecular weight (diffusible dyes) by both lymphatics and blood capillaries. In the same chapter it was mentioned that the synovial membrane has no capillaries or lymphatics which are freely

accessible from the joint cavity; a covering consisting of connective tissue is always interposed between the cavity and the endothelial structure. This implies that the passage of both solute and solvent must take place through this layer of connective tissue before any absorption can occur via the lymphatics or blood capillaries.

#### **The Effect on Joint Absorption of Exercise, Massage and Intraarticular Hydrostatic Pressure**

BRAUN (1894), KROH (1908), MÜLLER and LAUBER (1932), BAUER *et al.* (1933), and ALLEN (1935) have all found that passive movements or massage of joints promote the absorption from joint cavities. MÜLLER and LAUBER (1932), after experiments on one dog, report that compression of one knee joint did not increase the absorption of injected water-soluble, radio-opaque material, as compared to the uncompressed knee joint. KASAHARA (quoted from MÜLLER and LAUBER) reportedly obtained a greater absorption from joint cavities when the intraarticular pressure was raised.

All the above papers are agreed that joint motion and massage increase the absorption from joints, but none of them permit any conclusions to be drawn as regards the effect of intraarticular hydrostatic pressure.

Earlier literature, including absorptive studies, on the physiology of articular structures has been reviewed by BAUER *et al.* (1940), and in part by EFSKIND (1941 a, b).

#### **Factors Affecting the Movement of Fluid and Soluble Matter in Connective Tissue other than Synovial Membranes**

I. THE INFLUENCE OF BLOOD CIRCULATION ON FLUID EXCHANGE. Since no quantitative data on the exchange of fluid through the synovial membrane are known, this phenomenon must be discussed in the light of knowledge gained through experiments with other types of connective tissue.

STARLING's classical hypothesis (1896), based on his investigations into fluid absorption from connective tissue spaces, states, assuming the outward and inward fluid transfer to take place at respectively the venous and arterial end of the same capillary vessel, that the filtration of fluid and its absorption are governed by the following factors:

1. Oncotic pressure differences between the fluid in the blood capillaries and the surrounding tissues.
2. Hydrostatic pressure differences between the capillaries and the surrounding tissues.
3. The physical properties of the capillary membranes considered as mechanical filters.

PAPPENHEIMER and SOTO-RIVERA (1948) have proved that STARLING's theory is valid with a very high degree of accuracy, by using amputated mammalian hind limbs as test objects.

However, the above experiments on limbs where nervous control had been eliminated do not exclude the possibility that in the intact animal factors other than those listed may contribute to the maintenance of fluid exchange through capillary membranes.

One such factor would be the type of fluid movement through capillary membranes proposed by ZWEIFACH and co-workers, in whose opinion vasomotor adjustments of the areas of outward filtration and inward absorption in the capillary bed are of some importance. In contrast to STARLING's theory which presupposes that filtration and absorption occur in the same capillary, ZWEIFACH and co-workers claim that filtration and absorption, respectively, take place in anatomically individual vessels in the capillary bed.

Briefly, the mechanism of the above theory is as follows: In tissues where the volume of flow is fairly constant the capillaries are not perceptibly organized, but tissues where the nutritional demand varies with the functional activity exhibit a well defined pattern. The skin, for example, and the muscular and gastrointestinal systems are such tissues. Whenever the tissue is inactive the flow of blood is restricted to preferential channels; during tissue activity, however, the blood flow is widespread through the capillary network. The preferential vessels have been termed thoroughfare or "a-v channels", their proximal portions metaarterioles. The proximal portion and its precapillary sphincteric offshoots are muscular and change calibre spontaneously following activity of the muscular cells. This motor activity of the vessels is termed vasomotion and consists of a slow, intermittent, partial constriction or relaxation at intervals of about 30 seconds—3 minutes. The mechanism is thought to regulate the blood flow through the a-v channels and the "true" capillaries. The latter vessels are devoid of muscular elements and constitute the main type of vessels in the capillary bed. They start from the pre-



capillary offshoots of the metaarterioles or arterioles and rejoin the distal continuation of the a-v channels through inflowing tributaries. The "true" capillaries sometimes constitute interanastomosing systems.

#### Alleged Influence of Vasomotion on Fluid Exchange.

The influence of vasomotion on fluid exchange in the capillary bed can be summarized as follows: The mean hydrostatic pressure is relatively low in the true capillaries, and this is due to the sphincteric action of the precapillary musculature. The flow through the a-v channels is more rapid than elsewhere in the bed; the drop in pressure between the arterial and venous end of this vessel is claimed to be of smaller magnitude than in the side branches; the a-v channel is thought to be the dominating organ for outward filtration; inward absorption on the other hand, should be brought about by the true capillaries.

This influence of vasomotion on fluid transfer which is caused by modifications in the ratio between hydrostatic and colloid osmotic pressures in the capillary bed is said to take place as follows:

1. Alterations in the time ratio between the duration of the constrictor and dilator phases of the precapillary sphincters. Obviously a predominance of the constrictor phase of the precapillary sphincters (increased vasomotion) would tend to decrease the hydrostatic pressure in the true capillaries and facilitate the absorption of tissue fluid to these vessels, provided they are not bloodless.

2. Alterations in the rate of intermittent change in the calibre of the a-v channels. Since to vasomotion is attributed a power of diminishing and sometimes interrupting the flow in the a-v channels where an outward filtration of fluid is said to take place, an increased vasomotion would tend to decrease the transfer of fluid from the vascular bed.

Briefly, therefore, in consideration of 1 and 2, increased vasomotion tends to promote the absorption of fluid from the tissues.

Of course, the hydrostatic pressure in the capillary bed is also dependent on the state of constriction and rhythmic activity of the terminal arterioles outside the capillary bed, the consequences of which on the transfer are obvious in the light of Starling's theory. A decreased or ceased vasomotion, possibly combined with dilatation of the terminal feeding arteriole, tends to flush the entire capillary bed,

giving an increased outward filtration culminating in edema, presupposing a relatively unchanged arterial blood pressure.

Increased vasomotion is said to take place after acute hemorrhage, sympathetic stimulation, intravenous administration of adrenalin, angiotonin and adrenal cortical extract. Diminished vasomotion is a concomitant of elevated body temperature, lowered body temperature, direct trauma, increased vital activity, prolonged anoxia, denervation. Reportedly, increased vasomotion decreases lymph flow; diminished vasomotion increases it. (ZWEIFACH 1940, CHAMBERS and ZWEIFACH, 1944, ZWEIFACH *et al.* 1944, CHAMBERS and ZWEIFACH 1947 a, review of the literature by CHAMBERS and ZWEIFACH 1947 b).

The influence of vasomotion on fluid absorption in connective tissue has never been directly measured. MC MASTER (1941 a, b) observed, however, an intermittent absorption of fluid in dermal connective tissue in the ears of mice and rabbits. He interpreted this intermittence to be interrelated with the blood circulation since it could not be demonstrated in dead animals. In hyperemia, reactive and reflex-induced, an increased, intermittent inflow of fluid was observed as long as no edema existed, in which case there was intermittent outflow of fluid from the tissues. (MC MASTER 1941 b).

Summarizing, the exchange of fluid through the synovial membrane is probably governed by the factors entering in the classical theory of Starling, perhaps modified by the existence of a vasomotor mechanism as proposed by ZWEIFACH and co-workers. To these mechanisms, which merely apply to fluid transfer between the joint cavity and the blood vessels, must be added the following mechanism. The hydrostatic pressure of the injected test fluid used in the present investigation may be able to force fluid out of the joint cavity into the connective tissue itself. With an isotonic electrolytic solution as test fluid, this would mean that a fraction of the fluid which was absorbed from the joint cavity may be forced outwards by a mechanism quite apart from the blood circulation.

II. THE TRANSPORT OF FLUID AND SOLUBLE MATTER THROUGH CONNECTIVE TISSUES OTHER THAN SYNOVIAL MEMBRANES. Earlier works on the permeability of the connective tissue of the skin have been reviewed by DURAN-REYNALS (1942).

The results obtained on dermal connective tissue by means of

intradermal wheals injected at very high pressures cannot, however, give fully adequate information about the physiological mechanism governing the transport of fluid and soluble matter in connective tissue where normal pressure conditions prevail, i. e. where the formed elements are not forced apart by the injected fluid. Therefore only those papers will be discussed where physiological pressure conditions were employed.

Using a method whereby vital dyes could be injected into the dermal connective tissue in the ears of rabbits, MC MASTER and PARSONS (1938) concluded:

1. Perfusion of the ears with blood using pulsating perfusion pressure increased the spreading rate of the dye in comparison to experiments where the perfusion pressure was constant.
2. Not only the spreading of the dye but also its absorption from the ear was increased when pulsating pressure was used.
3. During edema formation the spreading of dyes was greater than in normal ears when pulsating pressure was used.

Using the ears of mice as test objects, PARSONS and MC MASTER (1938) demonstrated that:

1. Hyperemia increases the spreading of dyes through the tissues.
2. Delivering the animals of between 2.5—4 per cent of blood expressed as body weight decreases the spreading area as compared with normal controls.
3. Very little spreading of the dye could be observed in animals just killed (ether or chloroform).
4. During edema formation the spreading of the dye was greater than in normal controls.
5. In manifest edema in either living or dead animals the spreading of the dye was about the same and not greater than in normal living control animals.
6. In actively moving unanesthetized animals the spreading of the dye was increased in comparison to anesthetized controls. Intermittent, pulsating pressure applied to the ears of unanesthetized mice (pressure range 2—8 cm  $H_2O$ ) produced an enormous increase in the spreading area.

These experiments lead to the conclusion that the spreading of dyes along or between tissue elements, not widely separated would be favored by such mechanical forces as tend to rub or squeeze the formed elements together.

All the experiments involving increased spreading were interpreted with this hypothesis in mind. When edema was being formed this "squeezing effect" was considered possible; when edema had already developed this rubbing action of the pulse was impossible owing to the separation of the formed elements by edema fluid.

In an extension of the above investigations MC MASTER and PARSONS (1939 a, b) found that the spreading of dye in the connective tissue of mice took place along the fibrils, serving as conductors. Rubbing or squeezing of these thin perifibrillar films greatly increased the spreading of the dye along the fibres. In normal connective tissue no free movable fluid could be detected. In edema the appearance of the dye was different in so far as it appeared in the tissues as diffusely coloured clouds that could be freely moved.

The possibility that the results obtained with respect to the perifibrillar movement of dyes were caused by their staining properties was thought to be excluded by the use of several chemically very different dyes.

In summary, the transport of dyes through connective tissue partly takes place in perifibrillar films and is greatly promoted by certain mechanical effects. In truly edematous tissue these mechanical effects apparently play a minor rôle; here the spreading of dye is likely to take place only by diffusion.

In tissues where edema is being formed and where the resistance to flow is accordingly lessened (MC MASTER 1941 c) the afore-mentioned mechanical forces are still effective, resulting in an increased spreading of the dye.

Whether or not the perifibrillar movement of dyes in connective tissue can be related to an affinity of certain acid azo dyes to the fibrillar elements in connective tissue (HELANDER 1945) or to the elastin affinity of several acid dyes, especially azo dyes, (KIYONO and AMANO 1937/1938) will be discussed.

The possibility exists that in early stages of diffusion through the intercellular gel of the connective tissue the dyes are not perceptible. Affinity to the fibrillar elements, causing an insoluble compound between fibrils and dye, would then tend to cause a concentration gradient between the tissue and the fibrils, accentuating the colouration of the fibrils. At this stage one would get the impression of the dye moving along the fibres. When the concentration of the dye in the gel becomes sufficiently high the latter becomes coloured to

the same extent as the fibres, the surface of which are saturated with colouring material. The above state of affairs, however, seems to be rather improbable, because as the second phase in the transport of dye MC MASTER and PARSONS (1939 a, b) found a broadening of the perifibrillar films, indicating that a diffusion of dyes from the fibres took place. This broadening was evident before any free movable fluid in the tissue could be observed.

#### **The Factors Governing the Movement of Fluid and Soluble Matter in Connective Tissue as Applied to the Problems Involved by Absorption from Joint Cavities**

The following considerations deserve attention:

1. The influence of blood circulation on the absorption of fluid from joint cavities.
2. The effect of blood circulation on the transfer of fluid and soluble matter through the synovial membrane.
3. The influence of the intraarticular hydrostatic pressure on the absorption of fluid and soluble matter from joint cavities.

1. The influence of circulatory changes in the synovial membrane on fluid exchange should be the same as in connective tissue elsewhere.

Lowering the hydrostatic pressure in the small blood vessels should facilitate the absorption of fluid from the joints.

The same effect would be obtained if the vasomotion increased. However, it is impossible to say whether an anatomical arrangement and a circulatory mechanism similar to those found by ZWEIFACH and co-workers in the mesenteria, skin and muscles of various mammals and amphibians also exist in the synovial tissues of the rabbit. MC MASTER's studies (mentioned above) seem to indicate that at least intermittent absorption takes place in normal connective tissue of the ears of mice and rabbits. This may be due to a mechanism of vasomotion in the sense of ZWEIFACH *et al.* but may also be explained by the intermittent function of the terminal arteriolar vessels in the rabbit ear (See CLARK 1943 for description and references) or by the rhythmic function of the below mentioned arteriovenous anastomoses.

In the view of the present author it is very difficult to explain the increased inflow of fluid into hyperemic ears of mice and rabbits

(Mc MASTER 1941 b) if not a vasomotor mechanism including a shunt arrangement is involved. As is well known hyperemia increases the mean hydrostatic pressure in the capillary bed, and, according to STARLING's hypothesis, this would tend to increase the net. transfer of fluid from the blood capillaries.

Applying to the theories of ZWEIFACH and co-workers the increased absorption of fluid in hyperemic tissue would be possible with an unchanged vasomotor frequency provided that the number of absorbing "true" capillaries was sufficiently increased. However, rabbit ears are well supplied with arteriovenous anastomose (CLARK and CLARK 1930, 1932, 1934 a; b; review of the literature on arteriovenous anastomoses by CLARK 1938), which are rhythmically active even in some stages of hyperemia. Supposing that most of them are dilated during hyperemia and a fraction of them are rhythmically active, the theoretical possibility exists that the hydrostatic pressure is actually lowered in blood vessels where exchange of fluid takes place, (DRINKER and YOFFEY 1941). In other words the absorption of fluid to them is facilitated in spite of the increased blood flow, which is shunted directly from arteries to veins. The rhythmic activity of some of the anastomoses may in Mc MASTER's experiments have been measurable as intermittent inflow.

Summing up, the intermittent absorption of fluid in the normal mouse and rabbit ear together with the increased intermittent absorption in hyperemic ears can theoretically be explained by two different mechanisms, vasomotion in the sense of ZWEIFACH and co-workers and/or rhythmic activity and dilatation of arteriovenous anastomoses in the connective tissue of the ears.

The existence of such mechanisms in the connective tissue of the synovial membrane in rabbit should give similar results. It is unknown whether arteriovenous anastomoses or the pattern of the capillary bed, permitting vasomotion, exist in the synovial membrane of the rabbit.

2. The described phenomena, increasing or decreasing the spreading of matter in dermal connective tissue, should be valid also for the absorption of colloids from joint cavities.

The effect of the pulsation energy transmitted to the tissues in connection with the interstitial transfer of matter must, however, be discussed at some length.

It has been mentioned above that hyperemia increases the rate

solution may increase or decrease depending on the number of absorbing "true" capillaries and frequency of vasomotion. The same effects as are caused by vasomotion in hyperemic synovial membranes would be caused by shunting the blood through arteriovenous anastomoses in hyperemic synovial membranes.

3. The effect of intraarticular hydrostatic pressure on the absorption of Ringer solution has been discussed in chapter III. It is impossible to decide how much of the increased absorption at raised perfusion pressures that was caused by increased seepage of fluid into the connective tissue or, respectively, by increased absorption to the blood capillaries. The effect of intraarticular pressure on the absorption of colloids will be discussed later in this chapter.

#### **Are Comparisons Justifiable between the Mode of Absorption and Transfer of Solutes and Solvents in Connective Tissues of the Skin and Synovial Membranes?**

Anatomical evidence justifying such comparisons has been mentioned in chapter I of this treatise. It is known of dermal connective tissue that substances of small molecular weight are absorbed mainly via the blood capillaries, colloids of a molecular weight matching that of hemoglobin (65,000—68,000) mainly by the lymphatic system (LEWIS 1921, FIELD and DRINKER 1931, BARNES and TRUETA 1941). Joint cavities exhibit no differences in the mode of absorption which places them apart from connective tissue (BAUER *et al.* 1933, RHINELANDER *et al.* 1939, ADKINS and DAVIS 1940; review of the literature by BAUER *et al.* 1940). In chapter III it was demonstrated that the resistance to flow of synovial membranes was of the same type as in dermal connective tissue in mice. EDLUND and LINDERHOLM (1947) have shown that a mercurial diuretic (salyrgan = mersalyl) increases the absorption of colloid and water from joints. This action of the drug is not due to any changes in the osmotic pressure of the blood (EDLUND and LINDERHOLM 1949 a) but to a "spreading factor" action of the drug as found by measurements of the permeability in the dermal connective tissue of rabbits (EDLUND and LINDERHOLM 1949 b). In joint membranes salyrgan, given in the same dosage as in the above papers of EDLUND and LINDERHOLM, causes a disappearance of the breaking point in the majority of cases, i. e. a decreased resistance to flow which is typical of the action of spreading factors (EDLUND and LINDERHOLM 1949 c). EDLUND and JUHLIN (un-

published experiments) have shown that a decreased permeability of the skin is in evidence after intraarticular burns. This diminished spreading of intradermally injected hemoglobin solution can be counteracted by administration of dibenamine, which agrees with the findings on joint absorption to be described in the next chapter of this paper.

Except for its contents of synovial mucin and plasma proteins joint fluid is in a state of diffusion equilibrium with blood and tissue fluid (cf. BAUER *et al.* 1940). The protein contents may easily be explained by the well known fact that blood capillaries are to a degree permeable to blood proteins. It is beyond the scope of the present paper to discuss the various theories concerning the site of formation of synovial mucin, but it is worth mentioning that some authors claim that synovial fluid is the liquid matrix of the connective tissue, lining an enlarged tissue space. (HUETER 1866, KEY 1928, KING 1935, COLLINS 1936, BERGER 1938). Thus, synovial mucin would correspond to the mucoid constituent of other connective tissues (HUECK 1920). VAUBEL (1935) maintains that mucin is the basic substance in synovial tissue. JOSEPH *et al.* (1948) from studies on diffusion potentials in dog synovialis concluded that in many cases the relative mobilities of ions corresponded to the values calculated for aqueous solutions, which have been confirmed for rabbit knee joints (EDLUND and TEORELL, unpublished experiments).

Summarizing, it may be said that in direct measurements of mode of absorption and transfer of solute and water there are no known differences between dermal connective tissue and synovial membranes. Indirect evidence — the composition of the synovial fluid and studies on diffusion potentials across synovial membranes — seems to indicate that no functions as regards selective permeability or absorptive and excretory powers of synovial membranes make them different from other connective tissue in which the exchange of fluid and soluble matter is considered to take place as the result of interaction between the various forces described earlier in this chapter.

The theoretical consideration remains that part or parts of the absorption from joint cavities may take place through the articular cartilage. Little is known about the mode and rate of transfer of fluid and solutes in cartilage. (Cf. BAUER *et al.* 1940.) INGELMARK and EKHOLM (1948), INGELMARK (personal communication) have



shown that articular cartilage in rabbits rapidly increases in thickness in connexion with increased joint function. This was interpreted as an imbibition of fluid from the articular space or more probably from the marrow cavity in those areas where the bone marrow extends to the basal parts of the cartilage. If this swelling of the cartilage actually is caused by a mechanism which has anything to do with absorption of fluid from resting joints is impossible to state with certainty. The swelling may be due to altered physico-chemical properties of the cartilaginous gel caused by the mechanical stress and its attendant phenomena (increased metabolism, changes in pH, etc.). The present author maintains that no existing evidence shows that absorption, measurable with the methods used in the present study, of fluid and solute can take place through the cartilage.

#### **Discussion of the Present Author's Results**

The accuracy of the methods has been discussed under the various control experiments. Under the prevailing experimental conditions they seem to be valid. However, the values for the initial intraarticular hydrostatic pressure obtained by extrapolating to zero time are somewhat doubtful. The statistical evidence justifying plotting of the intraarticular pressure as a function of the square root of the time in minutes after the injection of the hemoglobin solution is valid within the range of the measurements. Thus, it can never be proved that the extrapolation to zero time gives the initial hydrostatic pressure. However, the results obtained by absorption studies where various degrees of joint flexion were employed compared with the established level of the breaking point in synovial membranes suggest that the error due to the extrapolation is insignificant. Moreover, the existence of a breaking point in the five perfusion experiments performed at a joint flexion of 60° support the opinion that "breaking" pressures are not induced by intraarticular injection of 0.85 ml of test fluid.

THE INFLUENCE OF INTRAARTICULAR HYDROSTATIC PRESSURE AND PREVIOUS JOINT EXERCISE ON THE ABSORPTION OF COLLOID AND FLUID FROM JOINTS. The data obtained in these respects in conjunction with the studies by EDLUND and LINDERHOLM (1947) on the effect of salyrgan on the absorption of hemoglobin and fluid from rabbit knee joints and the effect of the same dosage of salyrgan on the

TABLE 7.

0.85 ml 5.5 g per cent hemoglobin solution injected intraarticularly at 0' in the absorption tests, where the mean value from both joints in each animal = one experiment	A Degree of joint flexion: 0°	B Degree of joint flexion: 60°	C Degree of joint flexion: 90°	D Degree of joint flexion: 60°. Exercised animals	E Degree of joint flexion: 60°. Salysrgan-treated animals
1. The amount of hemoglobin absorbed in per cent of injected Test period 40 minutes.	$m=50.5$ $e=\pm 1.5$ $n=12$	$m=51.4$ $e=\pm 0.7$ $n=46$	$m=59.8$ $e=\pm 2.1$ $n=18$	$m=58.3$ $e=\pm 0.6$ $n=15$	$m=59.0$ $e=\pm 1.5$ $n=14$
2. The intraarticular fluid volume in ml at the end of the test period; 40 minutes	$m=0.586$ $e=\pm 0.014$ $n=12$	$m=0.602$ $e=\pm 0.009$ $n=46$	$m=0.456$ $e=\pm 0.029$ $n=18$	$m=0.468$ $e=\pm 0.012$ $n=15$	$m=0.431$ $e=\pm 0.020$ $n=14$
3. The concentration of hemoglobin in g per cent in the intraarticular fluid at the end of the test period; 40 minutes.	$m=3.96$ $e=0.08$ $n=12$	$m=3.78$ $e=\pm 0.03$ $n=46$	$m=4.20$ $e=\pm 0.09$ $n=18$	$m=4.24$ $e=\pm 0.09$ $n=15$	$m=4.45$ $e=\pm 0.07$ $n=14$
4. Breaking point in cm H <sub>2</sub> O. (See table 1)		$m=9.55$ $e=\pm 0.58$ $n=20$		$m=7.98$ $s^2=4.34$ $n=5$	$m=12.66$ $s^2=9.33$ $n=7$
5. $\frac{dF}{dP}$ (ml/min/cm) · 10 <sup>6</sup>					
a. for pressures below the breaking point		$m=71$ $e=\pm 9.5$ $n=20$		$m=77$ $s^2=3752$ $n=5$	$m=103$ $s^2=882$ $n=7$
b. for pressures above the breaking point		$m=421$ $e=\pm 49$ $n=20$		$m=172$ $s^2=3120$ $n=5$	$m=462$ $s^2=27182$ $n=7$
c. in perfusion experiments not giving any breaking point				$m=231$ $s^2=2428$ $n=8$	$m=287$ $s^2=7872$ $n=8$
6. $\frac{dP}{dV\overline{T}}$ (cm/V $\overline{\text{min}}$ )					
Mean of regression coefficients calculated from the individual experiments where the intraarticular hydrostatic pressure was measured during the test period	$m=-0.612$ $e=\pm 0.044$ $n=13$	$m=-0.464$ $e=\pm 0.044$ $n=16$	$m=-1.108$ $e=\pm 0.108$ $n=15$	$m=-0.369$ $e=\pm 0.084$ $n=12$	

(Table 7 Cont.)

0.85 ml 5.5 g per cent hemoglobin solution injected intraarticularly at 0' in the absorption tests, where the mean value from both joints in each animal = one experiment	A Degree of joint flexion: 0°	B Degree of joint flexion: 60°	C Degree of joint flexion: 90°	D Degree of joint flexion: 60°. Exercised animals	E Degree of joint flexion: 60°. Salysan-treated animals
7. $\frac{dP}{dV\sqrt{T}}$ (cm/ $\sqrt{\text{min}}$ ) of the oncotic pressure of the hemoglobin solution in the joint cavities during the test period		$m = -1.197$ $e = \pm 0.074$ $n = 1$			
8. Initial hydrostatic pressure in cm H <sub>2</sub> O after injection of hemoglobin solution. Calculated from the individual experiments by extrapolation to zero time	$m = 7.07$ $e = \pm 0.39$ $n = 13$	$m = 5.38$ $e = \pm 0.43$ $n = 16$	$m = 13.03$ $e = \pm 0.97$ $n = 15$	$m = 5.42$ $e = \pm 0.67$ $n = 12$	

Table of differences:—

Differences	Numerical values of differences	<i>t</i>	<i>p</i>
1 C minus 1 B	+ 8.4	4.90	<0.01
2 C » 2 B	— 0.146	6.26	
3 C » 3 B	+ 0.42	5.37	
1 D » 1 B	+ 6.9	5.20	
2 D » 2 B	— 0.134	7.12	
3 D » 3 B	+ 0.46	5.95	
1 E » 1 B	+ 7.6	4.58	
2 E » 2 B	— 0.171	7.77	
3 E » 3 B	+ 0.67	8.71	
5 <sub>c</sub> D » 5 <sub>a</sub> B	+ 160	8.60	
7 B » 6 B	— 0.733	8.50	
8 A » 4 B	— 2.48	3.04	
8 B » 4 B	— 4.17	5.50	
8 C » 4 B	+ 3.48	3.24	
8 D » 4 B	— 4.13	4.70	
8 A » 8 B	+ 1.69	2.85	

Signs:  $m$ =arithmetic mean  
 $e$ =standard error of the mean  
 $s^2$ =variance  
 $n$ =number of experiments  
 $t$ : for the statistical meaning of  $t$  see YULE and KENDALL (1946).  
 $p$ : the probability that the difference is caused by chance.  
1 B, 2 B, 3 B calculated from values in table 5.  
1 E, 2 E, 3 E from EDLUND and LINDERHOLM (1947).  
4 E, 5 E from EDLUND and LINDERHOLM (1949 c).  
7 B: the standard error given is that of the regression coefficient calculated from the total sample given in fig. 19.

resistance to flow in synovial membranes (EDLUND and LINDERHOLM 1949 c) are summarized in table 7. The animal materials and experimental methods used by EDLUND and LINDERHOLM have been identical to those presented in this paper.

From the table it is evident that in all animal groups where it may be suspected that the structural integrity of the synovial membrane is broken the absorption values are statistically identical. Compared to "normal" animals tested at 0° and 60° of joint flexion — which in turn are statistically identical — the animals first mentioned exhibit an increased absorption of both colloid and fluid.

As stated above it was impossible to decide whether the pre-exercised rabbits had a slight edema in the knee joints.

At initial hydrostatic pressures exceeding the breaking point the absorption of colloid and fluid is increased. The same may be said of animal groups in which the breaking point is eliminated by previous joint motion or the administration of salyrgan.

In the latter groups of animals the intraarticular hydrostatic pressure has been measured in preexercised animals and was found to be statistically identical to that in the normal control group. During the test period the intraarticular pressure in the salyrgan treated rabbits (EDLUND and LINDERHOLM 1947) was not measured. There is no reason to suppose, however, that salyrgan should cause increased hydrostatic pressures during the test period as compared with untreated animals at the same degree of joint flexion. It may be concluded that the increase of intraarticular hydrostatic pressure above the breaking point causes a structural derangement, at least in part of the animals at 90° of joint flexion. In this group the absorption values are statistically the same as in animal groups where the intraarticular hydrostatic pressure is measured and presumed to be the same as in "normal" controls. (Exercised and, respectively, salyrgan treated animals.) However, in the two latter animal groups the resistance to flow of the synovial membranes is altered, i. e. there is no breaking point in part of the material. Hence, it seems defensible to assume that the variously caused structural derangement is responsible for the increased absorption of colloid and fluid rather than the intraarticular hydrostatic pressure itself.

The conclusion drawn is that at two statistically different initial hydrostatic pressures *below the breaking point*, the pressure does not affect the absorption of colloid and fluid. At initial pressures *above the breaking point*, when the structural integrity of the synovial membrane must have been broken, the absorption values are of the same magnitude as in animal groups where the structural derangement only has been induced.

The five perfusion experiments made 40 minutes after injection of the hemoglobin solution would seem to suggest that the absorption of fluid containing colloid increases with increasing pressures, both below and above the breaking point. Since, at least in dogs, joint capsules have a length modulus of elasticity of about 300 kg/cm<sup>2</sup> (DANCKELMANN 1937), it may be that the rise in intraarticular perfusion pressure levels cause an increased gradient of pressure from the joint cavity, since the intraarticular pressure is not transmitted to adjacent structures outside the synovial tissue. The result of this is an increased outflow of indicator solution. On the other hand, in the absorption studies where the intraarticular pressure was adjusted by various degrees of joint flexion, the following chain of events may take place. The degree of flexion used must induce tension in the muscles and tendons surrounding the joint, differing for each degree of joint flexion. This increased tension must so affect the intraarticular hydrostatic pressure that an increased tension tends to raise the intraarticular pressure. The result of raised tension in, for example, the structure surrounding the joint capsule when the joint cavity contains fluid may be an elevated pressure in the synovial membrane. In turn this may affect the permeability of the synovial membrane in line with the reasoning about liquid flow through deformable media in chapter III.

Thus, the identical results obtained with respect to the absorption of colloid and fluid at two different pressure levels below the breaking point may be caused in the following ways:

1. Increased pressures in the joint cavity do not mean that an increased pressure gradient exists across the synovial membrane.

2. The permeability of the connective tissue surrounding the cavity may have altered when the pressure exerted on the membrane changed. This may imply that changes in permeability counteract the effects of altered pressure gradients on fluid transfer through the membranes.

3. The findings of Mc MASTER (1941 c) that at pressures below the breaking point the inflow of homologous serum in dermal connective tissue is not raised by increased pressures, may be valid. Since no determinations with hemoglobin solution as perfusion fluid were carried out this possibility cannot be excluded.

Since the absorption values are statistically the same in the groups where the resistance to flow of the membranes is altered by various

means independent of the prevailing intraarticular hydrostatic pressure, the explanation under 1 above seems to be the most likely. This is especially true because MC MASTER's findings with serum as perfusion fluid clearly indicate that there is an increased inflow above the breaking point. The fact that in all groups of animals where the structure of the membrane has been altered the net. absorption of fluid is increased more than the absorption of hemoglobin may be explained by the possibility that the colloid serves as «carrier» of the water (see p. 68). The oncotic pressure in the experiments where the normal structural conditions of the joint membrane existed are reproduced in fig. 19 p. 49. A tissue fluid with such oncotic pressure would even after subtracting the intraarticular hydrostatic pressure tend to make any absorption of fluid to the blood capillaries almost impossible (EDLUND and LINDERHOLM 1947). The results obtained with 90° joint flexion concerning the net. volume absorption may partly be explained by the increased intraarticular pressure counteracting the movement of water from the blood capillaries into the joint cavity.

Whether a slight edema existed or not in the synovial membranes of preexercised rabbits does not seem to be of great importance. Both salyrgan and exercise have the same effect on joint absorption and resistance to flow in synovial membranes. The increased hemoglobin absorption caused by these factors may be explained by the findings of MC MASTER and PARSONS as regards the transfer of dyes in connective tissues where a slight separation of the tissue elements has been produced, together with the possible effects of intraarticular pressure on the movement of indicator fluid through the membrane when the resistance to flow is lessened. For discussion of the possible mechanisms that may serve to explain the action of salyrgan on connective tissue see EDLUND and LINDERHOLM (1949 b).

#### **Some Theoretical Aspects on the Absorption from Joints of Fluids and Soluble Matter**

The driving forces for similar transport in other connective tissues and their application to absorption from joints have been discussed earlier.

It is known that no "free" tissue fluid seems to exist in normal connective tissue (CLARK and CLARK 1933, MC MASTER and PARSONS 1939 a, b). This implies that the water is bound in a gel. The rate

of diffusion of ordinary solutes is almost the same in the pure solvent as in a dilute protein gel in the same medium. This must mean that the gel contains "pores" of solvent in which fluid is retained by capillary effects (GLASSSTONE 1946). Thus, the theoretical possibility exists that in connective tissue of the synovial membrane transfer of water and electrolytes caused by diffusion and osmosis may take place throughout the membrane and not preferably along the fibrillar elements like dye transfer in another type of connective tissue. (Mc MASTER and PARSONS 1939 a, b). On the other hand, if hemoglobin partly is transported through the membrane along the fibres, or through it, by the mechanical forces mentioned earlier, there may take place the following chain of events, partly derived from the experiments in the present chapter.

The following is evident from tables 5, 6, 7, and figs. 12, 19.

1. The oncotic pressure of the intraarticular hemoglobin solution decreases steadily at a more rapid rate than the intraarticular hydrostatic pressure.

2. Hemoglobin absorption takes place throughout the test period.

3. The changes in intraarticular volume are greatest at the beginning and end of the test period.

Hence the effective oncotic force exerted by the intraarticular hemoglobin solution is insufficient to move fluid from the tissues and blood capillaries to the joint cavity at such a rate as to render constant or increase the intraarticular hydrostatic pressure and 1 or the intraarticular fluid volume; the "resistance" of the membrane is too high. The same "resistance" to water movement should be exerted when hemoglobin is conveyed through the membrane, i. e. it is only slowly diluted.

Where hemoglobin can go there must be possibilities for water to go. The solvent — water — for the hemoglobin when it passes through the membrane must come from somewhere. Supposing now that mechanical forces take part in the transport of hemoglobin through the membrane and that the "resistance" is high to water movement from the tissues and capillaries to the amount of hemoglobin being in transport through the membrane, then it is likely that part of the solvent water is taken from the water in the joint cavity. On the other hand it is possible that these mechanical forces — for reasons mentioned below — may cause no measurable net fluid transfer of e. g. Ringer solution. The same mechanical forces may

become measurably active in fluid transfer when a colloid is added to the joint fluid, because the solute — hemoglobin — has a net. transfer gradient directed from the joint cavity, and because part of the solvent water for the hemoglobin is taken from the joint fluid.

The most extreme consequence of this theory would be that more fluid is absorbed from the joint cavity when the intraarticular fluid contains hemoglobin than when it consists of an equiionic water solution containing no colloid. This may be briefly explained as follows. Under physiological conditions the filtration of fluid from the capillaries and absorption to them plus the lymphatic absorption are in equilibrium. This balance should only be slightly affected by introduction of Ringer solution in such an amount that the intraarticular hydrostatic pressure does not rise appreciably above the "tissue pressure" of the synovial membrane. The result should be a small net. absorption of fluid from the joint cavity, smaller than when hemoglobin was absorbed and served as a "carrier" of the water.

### Comment

The methods developed have made possible measurements of intra-articular hydrostatic pressure in and absorption of colloid and fluid from joint cavities under normal and altered structural conditions of the synovial membrane. The method developed of measuring joint absorption merely gives the amount of fluid and solute conducted out of the joint cavity but says nothing about other exchanges of solute and solvent. Nor is it possible to state whether the absorption to the blood stream of either solute or solvent takes place via the lymphatics or directly to the blood capillaries after passing the barrier of connective tissues interposed between the joint cavity and the blood and lymph vessels.

The hemoglobin solution did not affect the resistance to flow of synovial membranes. The hemoglobin and fluid absorption was studied at various levels of intraarticular hydrostatic pressure.<sup>1</sup> It was found that at two such pressure levels below the breaking point the absorption was not different. It was doubtful, however, if the method used to raise the intraarticular pressure — varying the degree

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<sup>1</sup> In such absorption studies the ideal intraarticular hydrostatic pressure would be the «tissue pressure» of the synovial membrane, but this has never been measured.



of joint flexion — actually increased the pressure gradient across the synovial membrane. Initial pressures above the breaking point resulted in increased fluid and hemoglobin absorption, but similar effects were obtained in animals where only the structural conditions of the synovial membrane were changed. The reservations made above as to joint flexion, hydrostatic pressure and pressure gradients must be kept in mind also in the latter case.

Thus, the only statement that can be made is that the structural conditions of the synovial membrane must be changed in order to increase the absorption of colloid and fluid from joint cavities by means of a physiological method of raising the intraarticular hydrostatic pressure, i. e. joint flexion. Since such structural disorders appear after previous joint exercise, the changes causing the breaking point to disappear in the exercised rabbits may possibly be a physiological consequence of stress, serving to promote fluid exchange in connective tissue.

Earlier findings on joint absorption are briefly reviewed and earlier papers dealing with the transfer of fluids and matter through tissues, especially connective tissue, are in the discussion related to the problems arising from the same phenomena in synovial membranes.

Presented evidence seems to indicate that transfer of fluid and soluble matter through synovial membranes are governed by the factors that regulate the same phenomena in connective tissue in general.

## CHAPTER V

### **The Effect of Subcutaneous Inflammation and Intraarticular Burns on the Absorption of Colloid and Fluid from Joints with Structurally Intact Synovial Membranes**

#### **The Effect of Dibenamine on Absorptive Changes thus Induced and on the Absorption of Ringer Solution from Normal Knee Joints**

DURAN-REYNALS and ESTRADA (1940) have found that intradermally injected staphylococcal and streptococcal cultures containing spreading factor produced successively smaller lesions when injected at short intervals. The phenomenon could be observed as early

as 30 minutes after the first inoculation. Concomitant with the reduced lesions caused by the second, third and subsequent injections of these bacterial species the spreading area decreased of intradermally injected dilute India ink. Such changes in the permeability of the skin were not caused by low virulent bacteria, except when the first lesion was very large or when spreading factor was added to the inoculum. DURAN-REYNALS (1942) suggested that this lowering of the permeability of dermal connective tissue in regions not inoculated with bacteria was not restricted thereto but applied to all connective tissue.

Commencing from some of the present author's observations as to the effect in rabbits of unilateral intraarticular burns on the absorption of colloid and fluid from the »normal» knee joint, EDLUND and JUHLIN (unpublished experiments) have established that intraarticular burns reduce the permeability of dermal connective tissue in rabbit. (Measured as spreading areas of intradermal wheals induced by injected hemoglobin solutions.) This reduction in permeability could be cancelled by dibenamine — which in itself is without effect on the permeability of dermal connective tissue.

### Experimental

With the object in view of investigating the effects of subcutaneous inflammations, intraarticular burns and dibenamine on the absorption of colloid and fluid from joint cavities, the following sets of experiments were made:

1. The effect of subcutaneously injected virulent bacteria on the absorption of hemoglobin solutions from joints.
2. The effect of unilateral intraarticular burns on the absorption of hemoglobin solutions from the non-lesioned knee joints.
  - 2 a. The effect of dibenamine on the absorptive changes caused by intraarticular burns. Experiments as under 2.
3. The effect of intraarticular burns on the absorption of Ringer solution from the »normal» knee joints.
4. The absorption of a test fluid, isoionic with the hemoglobin solution employed, from »normal» knee joints in not burned animals.
5. The effect of dibenamine on the absorption of Ringer solution from normal joints, with and without intraarticular burns in the contralateral knee joints.

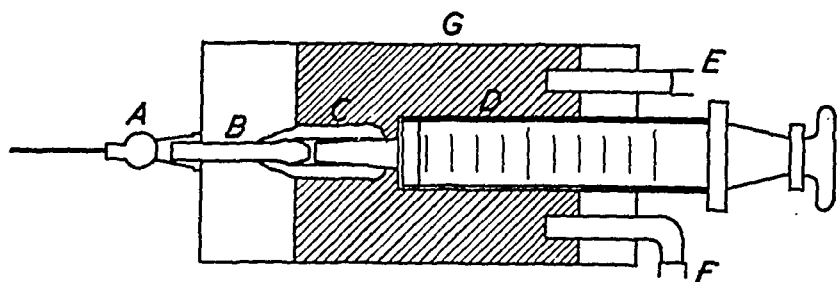


Fig. 20. Water-jacketed syringe. For description see text.

6. The effect of intraarticular burns on the intraarticular hydrostatic pressure induced by a given amount of test fluid injected into the normal joints in comparison with that in animals not burned.

7. The effect of dibenamine on the resistance to flow of normal synovial membranes.

8. The effect of intraarticular burns on the resistance to flow of the synovial membranes in the non-lesioned joints.

9. The effect of intravenously injected sucrose solution on the absorption from normal joint cavities of hemoglobin solutions.

### Methods

Subcutaneous inflammations were induced by injection in the nuchal regions of the animals of 2 ml of 24-hour old cultures of the staphylococcus strain described in chapter II. Intraarticular burns were caused by injecting 0.5 ml of 96° Ringer solution into the right knee joints of the anesthetized rabbits.

The latter injections were made (see fig. 20) with an all-glass syringe (D) mounted in a lucite water jacket through which flowed 300 ml/min. hot water, entering through F and escaping through E. The temperature of the circulating water was such as to impart a temperature of 96° to the contents in the syringe. By means of the rubber sleeve C and the adaptor B the syringe D was connectable to the intraarticularly inserted needle A.

Dibenamine, N,N-dibenzyl- $\beta$ -chloroethylamine hydrochloride, was administered intravenously in the marginal veins of the ears in doses of 10 mg/kg body weight as a 1 per cent solution in 0.9 per cent saline with sufficient 1 N HCl added to dissolve the drug. Such solutions have a pH of 1.5–2. In all cases the drug was injected during two minutes.

All experiments were made with a joint flexion of  $60^\circ$ . In the absorption experiments 0.85 ml of test solution was injected at the beginning of the absorption periods. In groups 1, 7, 9 the experiments began ten minutes after the end of the urethane injection. The hemoglobin solutions had a concentration of 5.5 g per cent (see p. 7 for data). The anesthetic, urethane, was administered as described in chapter II.

### Performance of The Various Types of Experiments

1. The bacterial culture was injected three hours before the absorption test began. Both knee joints of the same animal were used. The controls were animals which three hours earlier had been injected with 2 ml of Ringer solution in the nuchal region.

2. The urethane injection having been completed, hypodermic needles were inserted into the right knee joints of the rabbits. After the subsequent injection of 0.5 ml Ringer solution ( $96^\circ$ ) into the joint cavities the needles were stoppered. Needles were then inserted into the corresponding left joints, just before the beginning of the absorption tests, 30 minutes after the injections of Ringer solution. The controls had been given 0.5 ml  $39^\circ$  Ringer in the right knee joints as described above.

2 a. The dibenamine experiments were performed exactly as under 2 except that the drug was given only to animals burned with  $96^\circ$  Ringer solution. Dibenamine was given immediately before the beginning of the absorption tests.

3, 4, 5. These experiments were carried out exactly as under 2 and 2 a except that the test solutions were Ringer and an electrolytic solution equiionic with the used hemoglobin solutions. Here the washing fluid was 5.5 g per cent hemoglobin solution.

6. The intraarticular hydrostatic pressure during the test period was measured in the normal joints of animals subjected to contralateral burning 30 minutes before injection of 0.85 ml hemoglobin solution. The pressure was measured as described in chapter IV.

7, 8. The resistance to flow of the synovial membranes was determined as described in chapter III, the perfusion period at each pressure level being 4—6 minutes. The perfusion experiments started as soon as dibenamine had been given, i. e. 10 minutes after the end of

TABLE 8. In all absorption tests and in measurements of intraarticular hydrostatic pressures a volume of 0.85 ml test solution was injected intraarticularly. In test groups 1, 2, 11, 12 are the mean values from both joints in each animal = one experiment. In the other groups only the left knee joints were used in the tests.

Signs: $m$ = arithmetic mean $e$ = standard error of the mean $s^2$ = variance $n$ = number of experiments $t$ : for the statistical meaning of $t$ see YULE and KENDALL (1946) $p$ : the probability that the difference is caused by chance	A Hemoglobin absorbed in per cent of injected	B Intraarticular fluid volume in ml at end of test period	C Concentration of hemoglobin in g per cent at end of test period
1. Animals injected with virulent bacteria in the nuchal region 3 hours before the tests	$m=46.3$ $e = \pm 1.4$ $n=13$	$m=0.621$ $e = \pm 0.024$ $n=13$	$m=4.07$ $e = \pm 0.11$ $n=13$
2. Animals injected with Ringer solution in the nuchal region 3 hours before the tests. Test period in groups 1 and 2: 40'	$m=54.2$ $e = \pm 1.1$ $n=12$	$m=0.572$ $e = \pm 0.015$ $n=12$	$m=3.66$ $e = \pm 0.09$ $n=12$
3. Animals burned in the right knee joint with 96° Ringer solution <sup>1</sup> at 0'. Test period 30'—70' after burning	$m=41.2$ $e = \pm 1.2$ $n=16$	$m=0.657$ $e = \pm 0.027$ $n=16$	$m=4.24$ $e = \pm 0.11$ $n=16$
4. Animals treated as under 3 except that 39° C Ringer solution was injected in the right knee joint	$m=48.7$ $e = \pm 1.9$ $n=14$	$m=0.599$ $e = \pm 0.036$ $n=14$	$m=4.07$ $e = \pm 0.11$ $n=14$
5. Animals treated as under 3 except that dibenamine was given	$m=48.7$ $e = \pm 1.8$ $n=12$	$m=0.610$ $e = \pm 0.027$ $n=12$	$m=3.98$ $e = \pm 0.12$ $n=12$
6. Animals treated as under 3. Test fluid Ringer solution <sup>2</sup>		$m=0.658$ $e = \pm 0.020$ $n=12$	
7. Animals treated as under 4. Test fluid Ringer solution		$m=0.760$ $e = \pm 0.018$ $n=12$	
8. Animals treated as under 6 except that dibenamine was given		$m=0.623$ $e = \pm 0.027$ $n=12$	
9. Animals treated as under 7 except that dibenamine was given		$m=0.473$ $e = \pm 0.025$ $n=10$	

<sup>1</sup> Temperature of the Ringer solution in the water-jacketed syringe, the temperature of the solution when brought into contact with the synovial membranes is of course unknown.

<sup>2</sup> Washing made with hemoglobin solution. Sample for determination of hemoglobin concentration taken from the washing fluid after washing.

(Table 8 Cont.)

Signs: $m$ = arithmetic mean $e$ = standard error of the mean $s^2$ = variance $n$ = number of experiments $t$ : for the statistical meaning of $t$ see YULE and KENDALL (1946) $p$ : the probability that the difference is caused by chance	A  Hemoglobin absorbed in per cent of injected	B  Intraarticular fluid volume in ml at end of test period	C  Concentration of hemoglobin in g per cent at end of test period
10. Animals treated as under 4. Test fluid was an electrolytic solution <sup>1</sup> equiionic with the hemoglobin solutions		$m=0.733$ $e = \pm 0.029$ $n=13$	
11. Animals given sucrose solution at the beginning of the test period which was 20'	$m=44.7$ $e = \pm 1.0$ $n=15$	$m=0.549$ $e = \pm 0.013$ $n=15$	$m=4.73$ $e = \pm 0.06$ $n=15$
12. Animals not injected with sucrose, serving as controls to those in group 11. Test period 20'	$m=39.2$ $e = \pm 1.2$ $n=10$	$m=0.673$ $e = \pm 0.017$ $n=10$	$m=4.22$ $e = \pm 0.06$ $n=10$

<sup>1</sup> See chapter II for the composition of this solution.

Perfusion experiments. The experiments started 30 min after the injection of 96° C Ringer solution

Breaking point in cm H <sub>2</sub> O	$\frac{dF}{dP}$ (ml/cm/min) · 10 <sup>5</sup> for pressures below the breaking point
$m = 9.46$ $e = \pm 0.74$ $n = 10$	$m = 99$ $e = \pm 11$ $n = 10$

These values are not significantly different from the values obtained in experiments where no intraarticular lesions had been made (table 1, chapter III).

Measurements of intraarticular hydrostatic pressure. Hemoglobin solution was injected in the left knee joint 30 minutes after the injection of hot Ringer solution in the right

Initial hydrostatic pressure. Calculated by extrapolation to zero time	$\frac{dP}{d\sqrt{\text{min}}}$ (cm/ $\sqrt{\text{min}}$ ). Mean of regression coefficients
$m = 4.55$ cm H <sub>2</sub> O $s^2 = 1.94$ $n = 7$	$m = -0.350$ $s^2 = 0.0055$ $n = 7$

These values are not significantly different from the values ob-

tained in experiments where no intraarticular lesions had been made. (Values classed as 6 B and 8 B, table 7, chapter IV).

Table of differences:—

Table groups	1,	2	correspond to text group	1
»	»	3, 4	»	»
»	»	3, 5	»	»
»	»	6, 7	»	»
»	»	7, 8, 9	»	»
»	»	4, 10	»	»
»	»	11, 12	»	»
				2
				2 a
				3
				5
				4
				9

See under the heading "Experimental". The controls to the respective text groups are included in the table groups.

Differences between table groups	Numerical values of differences	<i>t</i>	<i>p</i>
1 A minus 2 A	- 7.9	4.15	} < 0.01
1 C » 2 C	+ 0.43	2.93	
3 A » 4 A	- 7.4	3.45	
3 A » 5 A	- 7.4	4.15	
6 B » 7 B	- 0.102	3.70	
8 B » 7 B	- 0.137	4.20	
9 B » 7 B	- 0.287	9.15	
9 B » 6 B	- 0.185	5.85	
4 B » 10 B	- 0.134	2.88	
11 A » 12 A	+ 5.4	3.38	
11 B » 12 B	- 0.124	5.80	
11 C » 12 C	+ 0.51	6.10	

the urethane injection and 30 minutes after the hot Ringer injection, respectively.

9. Immediately after the injection of hemoglobin solution into the first knee joint, 3 ml 50 per cent sucrose solution /kg body weight was injected during some minutes into the marginal vein of the ear. Hemoglobin solution was then injected into the other joint as described in chapter IV. The same hemoglobin solution was used in the experimental and control animals which were under treatment simultaneously.

### Results

The results appear in table 8, figs. 21, 22, 23, 24 and their respective captions. The following findings may be deduced from these results:

1. Animals subcutaneously injected with staphylococcal cultures exhibit lower absorption of hemoglobin from joint cavities than

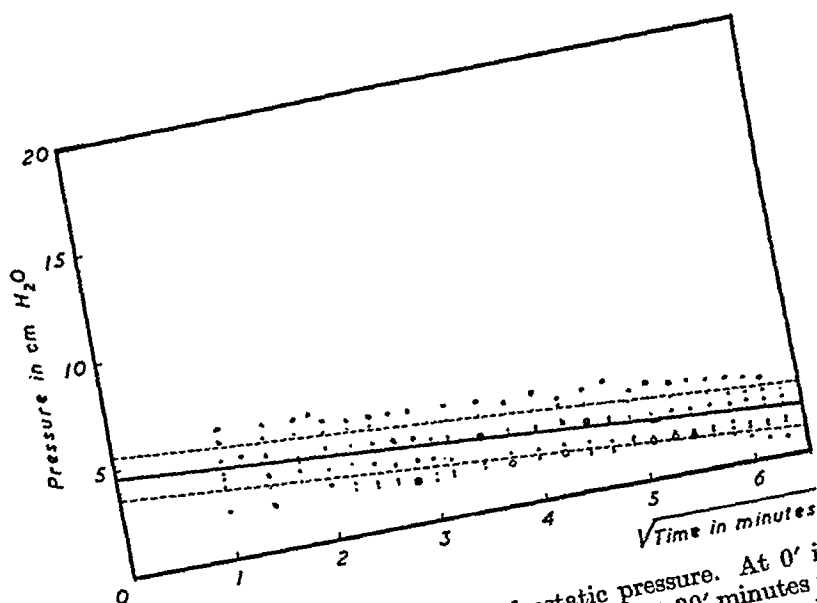


Fig. 21. Measurements of intraarticular hydrostatic pressure. At 0' injection of 0.5 ml Ringer solution (96°) in the right knee joints. At 30' minutes injection of 0.85 ml 5.5 g per cent hemoglobin solution in the left knee joints. Full line; regression curve, dotted line; standard error of estimate. Different signs in the same point of the graph indicate coinciding values. 7 experiments. Test of linearity:  $P < 0.01$ . Average correlation coefficient = 0.982. Range of correlation coefficients = 0.994—0.908. (For definition of terms and symbols see the legend to fig. 11.)

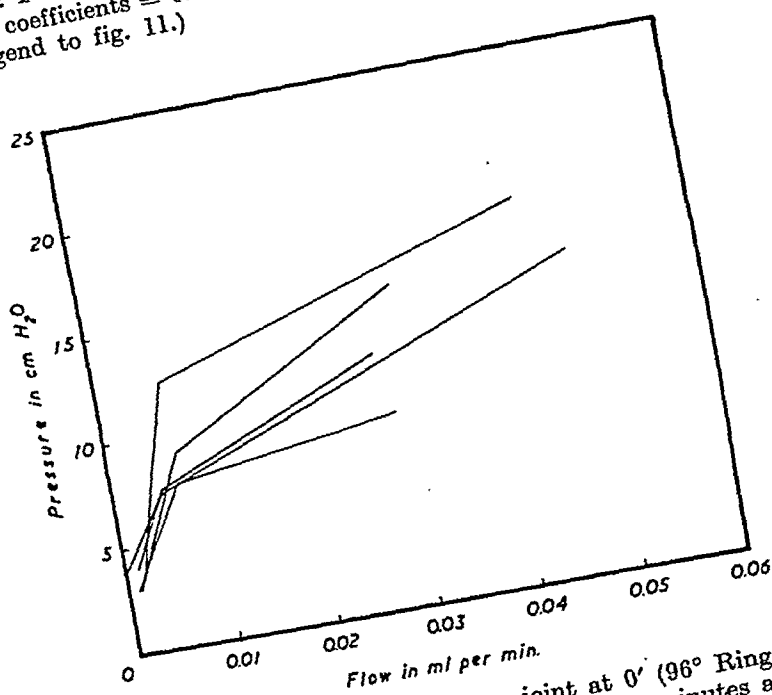


Fig. 22. Animals burned in the right knee joint at 0' (96° Ringer solution). Perfusion experiments began in the left knee joint 30 minutes after burning the right. Initial perfusion pressure about 3 cm H<sub>2</sub>O. Curves start at pressures where measurable inflow first could be registered and end at the highest used pressure. The two regression curves in each experiment were calculated according to the method of least squares.



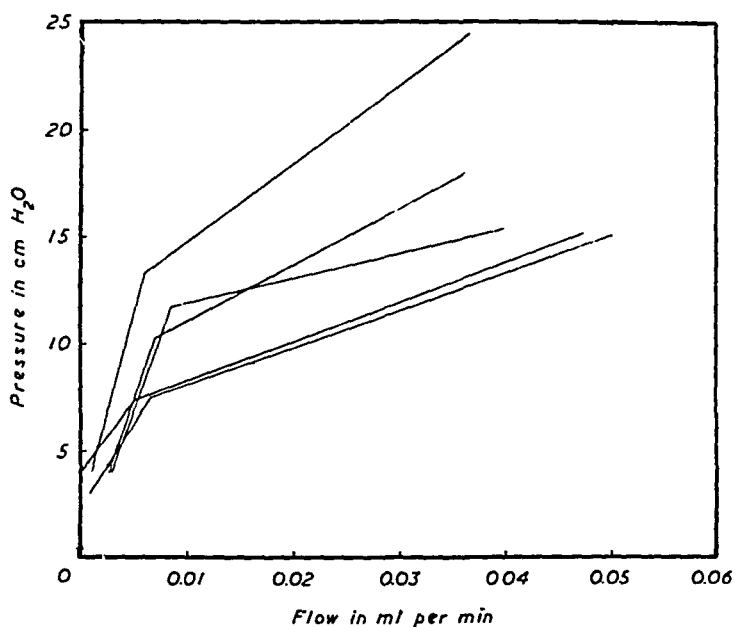


Fig. 23. Text, see fig. 22.

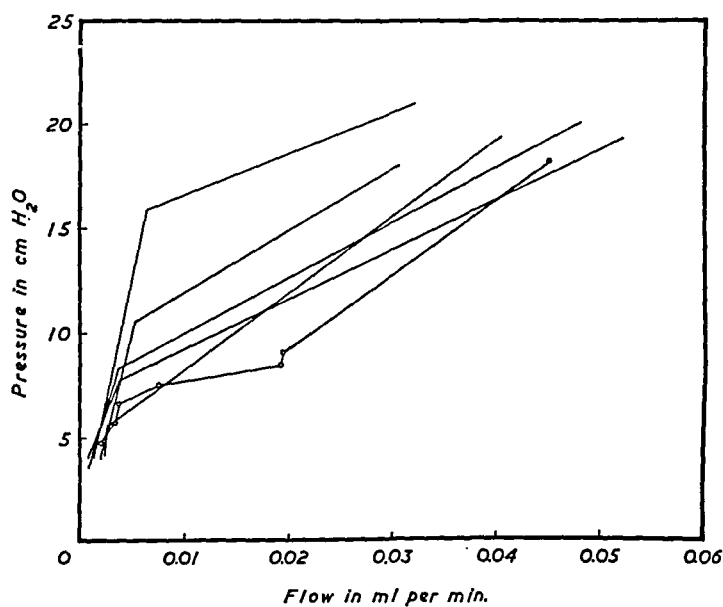


Fig. 24. Animals given dibenamine in connexion with the beginning of the perfusion experiments. Dibenamine has apparently no influence on the resistance to flow. In the five experiments where the breaking point could be calculated from the two regression lines, its mean value is 9.69 cm H<sub>2</sub>O. Average  $\frac{df}{dp}$  (ml/min/cm)  $\cdot 10^5$ , from these five experiments = 51. For obvious reasons the perfusion pressure levels and corresponding flow rates from the sixth experiment are not used in these calculations.

control animals. The hemoglobin concentration at the end of the test period is higher in the infected animals than in the controls.

2. Unilateral intraarticular burns reduce the absorption of hemoglobin from the »normal» joint cavities.

2 a. Such lowered hemoglobin absorption can be rendered void by dibenamine.

3. The absorption of Ringer solution is higher in the »burned» animals.

4. The net. volume absorption of an equiionic electrolytic solution containing no colloid is lower than of the same volume of 5.5 g per cent hemoglobin solution in the corresponding animal group.

5. Dibenamine increases the absorption of Ringer solution from the joints of unburned animals. In burned animals given dibenamine the absorption is significantly increased compared to normal and burned animals not given the drug.

6. Contralateral burns are without effect on the initial hydrostatic pressure caused by injection of 0.85 ml hemoglobin solution into the »normal» joint.

7. Dibenamine has no effect on the resistance to flow or the breaking point of synovial membranes.

8. Contralateral burns do not affect the level of the breaking point, nor the resistance to flow at pressures above or below the breaking point.

9. Sucrose, intravenously injected, increases the absorption of fluid and of colloid from joint cavities. In sucrose treated animals the hemoglobin concentration is higher at the end of the test period.

## Discussion

### Pharmacology of Dibenamine

Dibenamine is a powerful adrenolytic and sympathicolytic agent with persistent action. It blocks the effector cells to the vasoconstrictive action of epinephrine and the adrenergic transmitter. The adrenolytic action is more pronounced than the sympathicolytic (NICKERSON and GOODMAN 1947, DE VLEESCHOUWER 1947, BJÖRCK 1947, FOLKOW and UVNÄS 1948). In the doses used in the present investigation dibenamine causes no noticeable drop in the blood pressure of urethane anesthetized rabbits (EDLUND and JUHLIN,

unpublished experiments). The blocking effect was observed as early as 5 minutes after the injection, which agrees with the findings of the above authors.

#### **The Effect of Various Drugs and Injuries on the Permeability of Dermal Connective Tissue**

FAVILLI (1939, quoted from DURAN-REYNALS 1942) has found that intravenous and locally injected vasopressine inhibits the spreading of intradermally injected indicators admixed with testicular extract and snake venom, both the latter containing hyaluronidase. The effect of vasopressine is not apparent in excised rabbit skin. HOMBURGER (1945) has demonstrated that local and general reduction of the permeability of dermal connective tissue in rabbit could be induced by epinephrine and that this effect could be partially inhibited by ergotamine tartrate, which in itself decreases the permeability of the skin. CAHEN and GRANIER (1944) have shown that morphine (subcutaneous administration) decreases the permeability of dermal connective tissue in rat and rabbit when dilute India ink with or without hyaluronidase is used as indicator. One of the possible causes of this effect on the permeability of the skin advanced by these authors was the adrenergic action of morphine. MAYER and KULL (1947) have established that in rat the permeability of dermal connective tissue decreases when some antihistaminic drugs are administered to the animals; the same effect was obtained when hyaluronidase was added to the indicator — India ink. EDLUND and JUHLIN (unpublished experiments), using one of the antihistaminic drugs adopted by MAYER and KULL, have in normal rabbit skin verified the findings of the latter authors. EDLUND and JUHLIN have also found that this reduced permeability, caused by intravenously administered antistine (2-N-phenyl-N-benzoylaminomethylimidazoline) could be annuled by dibenamine, which by itself is without permeability effects. Furthermore, the action of antistine is (according to EDLUND and JUHLIN) not due to any antihistaminic or antihyaluronidase effects of the drug, the latter effect being suggested by MAYER and KULL.

Briefly it may be said of all the drugs mentioned, which decrease the permeability of dermal connective tissue, that theoretically, directly or indirectly, they may exert a vasomotor action. Vasopressine has a vasoconstrictive action as has ergotamine tartrate.

Some antihistaminic drugs are said to potentiate the action of epinephrine or increase the effect of sympathetic stimulation (for data and references see LOEW 1947, YONKMAN 1947). Anyhow, the fact that dibenamine annuls the decreased permeability of dermal connective tissue in rabbits treated with antistine strongly indicates that this effect of antistine is due to an adreno-sympathetic action. This is especially true since the action of dibenamine is not influenced by antihistaminic drugs (NICKERSON and GOODMAN 1947). In chapter IV of the present paper it was said that the transport of matter through connective tissue irrespective of the anatomical pattern of the capillary bed ought to decrease if the terminal arterioles are constricted. The effect of adreno-sympathetic stimuli<sup>1</sup> on the terminal arterioles should be vasoconstriction and increased vasomotion in the organs where the structure of the capillary bed has the arrangement described by ZWEIFACH *et al.* (see chapter IV of the present paper).

Thus the above drugs which have a vasoconstrictive action, may decrease the transport of soluble matter through connective tissue by reducing the amount of pulsation energy transmitted to the tissue; this in line with the theories of the previous chapter.

#### Remarks on the Release of Histamine from Injured Areas

LOOS (1931), LAMBERT and ROSENTHAL (1943), and DEKANSKI (1945, 1947) have shown that superficial skin burns increase the amount of recoverable histamine from the burned area in respectively rabbit, dog, mouse and cat. Working with inflammations induced by turpentine in the skin of rabbit, ZON *et al.* (1942) demonstrated an increase of histamine in the injured area. Thus, it may be that free histamine is absorbed to the blood stream from the injured areas. In cat and dog it is known that small histamine doses induce a discharge of epinephrine, leading to at least a temporary increase in systemic blood pressure (DALE 1920, KELLAWAY and COWELL 1922, HOGBEN *et al.* 1924, DALE 1926, FELDBERG 1929). A similar mechanism in rabbit would imply that the findings mentioned above of DURAN-REYNALS and ESTRADA may be imputed to »vasoconstrictive» action on the blood vessels, at least during the period when

<sup>1</sup> At least in animals with no known adrenergic vasodilator nerves, e. g. the rabbit (Burn 1938).

the first diminution of the lesion is observed. (For references on the influence on the permeability of dermal connective tissue of persistent inflammations see DURAN-REYNALS 1942.) On the other hand a »vasoconstrictive» action in burned animals or animals with dermal inflammations may as well be explained by increased adreno-sympathetic discharge caused by pain or fever following the injuries. (See GELLHORN 1943, BEST and TAYLOR 1945 for data and references.)

It being said that dermal connective tissue normally contains hyaluronidase (MEYER 1947: review of literature. MAYER *et al.* 1948) the general changes described above in skin permeability following various drugs and injuries might theoretically involve a mechanism influencing the activity of hyaluronidase and changing the state of aggregation and permeability of the intercellular gel. However, there is no evidence to support the existence of such a mechanism. (For papers and references on hyaluronidase inhibitors in blood see HECHTER and SCULLY 1947, HADIDIAN and PIRIE 1948, DORFMAN *et al.* 1948.)

SUMMARIZING, it may be said that the author has endeavoured to ascribe the decreased permeability of dermal connective tissue in the listed types of experiments to a circulatory function, constriction of the arterioles resulting in a decreased amount of pulsation energy transmitted to the connective tissue, diminishing the transfer of soluble matter (colloids) and (according to the theories in chapter IV) increasing the absorption of fluid to the blood vessels. With this in view the findings just mentioned will be associated with those on joint absorption given in the beginning of this chapter.

#### **The Relationship between the Experiments in this Chapter and the Theories on Joint Absorption in Chapter IV**

It must first be emphasized that neither unilateral burns nor dibenamine affect the resistance to flow of the synovial membranes in the unlesioned joints. The decreased colloid absorption in animals with staphylococcus infection or intraarticular burns, respectively, is interpreted by the author as though the lesions diminish the flow of blood through the normal synovial membranes, thereby lessening the mechanical forces supposedly partaking in the transport of hemoglobin. This being so, the absorption of fluid ought to be greater in the burned animals when the test fluid is

Ringer solution. Such was actually the case. Moreover, since dibenamine cancels the decrease in absorption of hemoglobin in the burned animals and has no effect on the permeability of the dermal connective tissue in normal rabbits, and since its blocking action is very specific, it seems likely that the increase in hemoglobin absorption in the animals given dibenamine is due to a blocking of the effector cells in the blood vessels to the action of epinephrine or any adrenergic transmitter that somehow is released by the injury. Accordingly dibenamine would tend to increase the blood flow in the synovial membranes of burned animals. However, as the blood pressure and the cardiac output in the burned dibenamine-treated animals is unknown, nothing definite can be said as to the cause of the increased absorption of Ringer solution in these animals. In the unburned animals given dibenamine the blood pressure ought to be the same as in the control animals (in parallel with the findings of EDLUND and JUHLIN reported earlier). It is, however, impossible to say if there is hyperemia of the synovial membranes in the dibenamine-treated animals as long as the cardiac output is unknown; if it were known the general degree of arteriolar dilatation could be approximated.

In any case, the decreased absorption of hemoglobin and the increased absorption of Ringer solution, respectively, in the burned animals cannot be due to any differences in intraarticular pressure during the test period as compared with the "normal" values given in table 7, p. 63.

The absorption tests carried out on animals injected with sucrose indicate that the dehydration of the tissues surrounding the joint has no reducing effect on the absorption of hemoglobin from joint cavities. The effect of the sucrose injection was that the absorption increased; of fluid greatly and of hemoglobin slightly.

MAC MASTER and PARSONS (1939 a) claimed tissue dehydration as a possible cause of the decreased transfer of dyes found by them in connective tissue of bled mice. LINDERHOLM (personal communication) has found a decreased permeability in the dermal connective tissue of rabbits subjected to dehydration by means of intraperitoneal injections of glucose solutions. Thus the decreased permeability of joint membranes and dermal connective tissue after various drugs and injuries may be due to tissue dehydration. This may be supported by the present author's findings that Ringer solution is

more rapidly absorbed from the not lesioned joints in burned animals, which is interpreted to be due to increased absorption of fluid to the blood vessels. However, it may be concluded that tissue dehydration induced by intravenous injection of sucrose, which causes an increased blood volume (ELLIS and FAULKNER 1939), by no means reduces the absorption of hemoglobin from joint cavities. The difference between the experiments carried out by the present author and by McMASTER and PARSONS is that their tissue dehydration was attended by a reduced blood flow, volume and pressure, thereby decreasing the amount of pulsation energy transmitted to the tissues. In the experiments of LINDERHOLM the blood volume decreases during the experiments (compare MELLORS *et al.* 1942), possibly resulting in the same circulatory effects as in the experiments of Mc MASTER and PARSONS and thereby giving a reduced skin permeability.

### Comment

In this chapter it has been shown that intraarticular burns and subcutaneous inflammations<sup>1</sup> decrease the absorption (as measured by the method described in chapter IV) of hemoglobin from joint cavities with structurally intact synovial membranes. In animals subjected to intraarticular burns the absorption of Ringer solution from the unlesioned joint cavities is greater than in the controls. The lowered absorption of hemoglobin in the burned animals could be raised to the same level as in the unburned controls by administration of dibenamine. In the burned and not burned animals dibenamine increased the absorption of Ringer solution. Dibenamine did not influence the resistance to flow of synovial membranes.

The present author tried to find a common cause for some of the data in the literature on permeability changes in dermal connective tissue following various drugs and injuries, and for the decrease

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<sup>1</sup> In this case it is unlikely that the decreased hemoglobin absorption was due to a structural derangement of the synovial membranes induced by septic metastasis. Routine testing of the "washing fluid" to detect intraarticular hemorrhage showed the same average number of leukocytes in the experimental and control groups. Moreover, the absorption of hemoglobin from joint cavities is increased in early inflammatory states (EDLUND 1948).

and increase, respectively, in the absorption of hemoglobin and Ringer from the joint cavities without lesions in animals with subcutaneous inflammation and intraarticular burns.

This common cause would be arteriolar vasoconstriction<sup>1</sup> due to different agents but having the same effect, viz., to diminish the amount of pulsation energy transmitted to the tissues and to augment the quantity of fluid absorbed to the blood stream.

The author's results seem approximately to fit the theories advanced in chapter IV. The effect of dibenamine on the absorption of Ringer solution in animals with and without intraarticular burns may be attributed to hyperemia in the synovial membranes caused by the drug; the hyperemia not being sufficiently pronounced to increase the hemoglobin absorption in the burned animals. The above findings with respect to Ringer absorption and dibenamine would then be in line with Mc MASTER's (1941 b), who has demonstrated an increased absorption of fluid in hyperemic connective tissue. If dibenamine induces hyperemia in the synovial membranes of Ringer-tested animals (which is unknown), the increased absorption of Ringer implies (for reasons see p. 57) that there is probably a shunt arrangement in the capillary circulation of rabbit synovial membranes (in animals without intraarticular burns the drug ought not to affect the blood pressure).

However, it must be mentioned that the validity of the above theories cannot be proved by the methods used here. The only way of verifying the theories is to investigate the absorptive properties, the anatomical arrangement, and the reaction to stimuli of the smaller blood vessels in different types of connective tissue.

Hence, it is evident that by studying the permeability of the unlesioned connective tissue in animals subjected to injuries a true picture will not necessarily be obtained of the permeability properties of "normal" connective tissue.

The parallelism between the changes in the permeability of dermal connective tissue and the absorption of colloids from joint cavities following subcutaneous inflammations and intraarticular burns

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<sup>1</sup> Obviously vasoconstriction causing total ischemia in the tissue concerned must decrease the transfer of colloids and completely invalidate the absorption of fluid to the blood capillaries. This may have been the case in some of the above experiments wherein the indicator fluid was admixed with vasoconstrictive agents.



indicates that, in these respects, synovial membranes are similar to connective tissue elsewhere.

The theory in chapter IV (p. 68), stating that the net volume absorption of hemoglobin solution may exceed that of an equiionic solution, was verified.

## CHAPTER VI

### **The Effect of Desoxycorticosteroneacetate on the Absorption of Hemoglobin and Fluid from Joints with Normal and Lesioned Synovial Membranes**

The effect of the cortical hormones of the suprarenal gland on the permeability of the blood capillaries has not been settled. The following review of literature will deal only with papers on capillary permeability in animals with intact suprarenal glands or with perfusion experiments on isolated hind limbs of animals. The literature on the physiology of the cortex hormones has been reviewed by SWINGLE and REMINGTON (1944).

The method most commonly used of studying the effects on permeability of these hormones has been to determine the seepage of intravenously injected vital dyes into local inflammatory lesions in the skin of animals and to note the action of various locally introduced agents on the amount of dye escape. However, this method is open to criticism (RIGDON 1940) since the stainability of the affected tissue seems to be altered by the induced lesions, so that the dye is accumulated in the tissue elements.

MENKIN (1940 a), using extracts of adrenal cortex as hormonal preparation and leukotaxine as irritant, has demonstrated that dye seepage into the lesions could be reduced by the glandular extract. Availing themselves of the same irritant and method, FREED and LINDNER (1941) have found that corticosterone and extracts of the cortex lower the accumulation of dye in the lesions — something that desoxycorticosteroneacetate (Doca) suspended in physiological saline does not do. With peptone as the irritant SCHLESER and FREED (1942) have found that cortical extract, unlike other hormonal preparations tested, decreases the accumulation of dye in the lesions.

MENKIN (1942) claims that FREED and LINDNER's negative results with Doca were due to the insolubility of Doca in water, since he found that Doca as well as other cortical hormones dissolved in sesame oil decreased the seepage of dye into injured areas when leukotaxine was used as irritant. GRAHAM (1943) has verified MENKIN's results by, unlike the previously mentioned authors, injecting the hormones "cortin" and Doca intramuscularly, the irritant being carbon arc irradiation of the skin. With perfused hind limbs of frogs as test objects, HYMAN and CHAMBERS (1943) report decreased capillary permeability as the effect of water soluble substances from the suprarenal cortex. Using perfused hind limbs of guinea pigs and frogs, JOSEPH and PALMER (1946) found cortical hormones to be without effect on capillary permeability. COPE and MOORE (1944) find no effect of adrenal cortical extracts in burned dogs after the burning procedure; they measured the concentration of radioactive brominated diazo dyes in the lymph and blood, thereby drawing certain conclusions about the capillary permeability.

Evidently the above publications do not permit any definite conclusions as to the permeability effects of the cortical hormones. An investigation into the effects of Doca on the permeability of normal and injured connective tissue, i. e. synovial membranes would therefore be rather interesting.

### Methods

The animal material, anesthetic, test solutions, and methods of measuring resistance to flow and absorption of hemoglobin and fluid are the same as are described elsewhere in this paper. The degree of joint flexion was 60° in all animals.

In some animal groups the irritant was the supernatant of a 24-hour culture of the staphylococcus strain used in the foregoing chapter. Before use the supernatant was filtered through a Seitz' filter and stored for 3 months at -20° C. Prior to injection into the joint cavities it was diluted 1:4 with 0.9 per cent saline. In other animal groups the lesions were caused by intraarticular injection of hot Ringer solution of 70° and 80°, respectively, with the aid of the water-jacketed syringe described in chapter V. 0.5 ml of the irritant was always injected immediately the needles had been inserted intraarticularly which was as soon as possible after the urethane injec-

tions. The groups in which both knee joints of the animals were used had a lapse of 5 minutes between the injections into the right and left knee joints. In all groups the absorption respectively the perfusion tests commenced 30 minutes after the induction of the lesions. The hormone<sup>1</sup> used was desoxycorticosteroneacetate dissolved in sesame oil, 5 mg/ml. The same preparation was used throughout.

All of the control groups were given sesame oil in amounts and at times corresponding to the Doca treated animals. In the absorption experiments 2.5 mg/kg Doca (or sesame oil to the controls) was given intramuscularly in one foreleg 45 minutes before lesioning. The procedure was repeated 45 minutes after lesioning in the other foreleg. To facilitate absorption both sites of injection were massaged for one minute. In the perfusion experiments 5 mg/kg of Doca or sesame oil was given intramuscularly in one foreleg 45 minutes before intra-articular injection of the irritant. The sites of injection were massaged for one minute.

Before the perfusion or absorption experiments were started the fluid remaining in the joint cavities was aspirated. It has been demonstrated (EDLUND, unpublished) that such aspiration is complete, viz. in animals with and without previous injections into the joint cavities the dilution of an intraarticularly administered test solution will be the same.

### Experimental

The following types of experiments were performed:

1. Absorption tests on animals treated with Doca and intraarticularly injected with the diluted staphylococcus filtrate. As in all the groups to follow the indicator used was 0.85 ml of a 5.5 g per cent solution of hemoglobin. The test period was 40 minutes in these as in all the subsequent absorption tests. The irritant was injected after the end of the urethane injection as soon as the needles were in place.

2. Same as 1 excepting that sesame oil was given instead of Doca.

3. Absorption tests as described above on animals given Doca only. Absorption tests began 30 minutes after the end of the urethane injection. No injuries made.

4. Absorption experiments as under 3 on animals given sesame oil only.

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<sup>1</sup> (Kindly supplied by AB Pharmacia, Stockholm).

5. Absorption tests as under 1 on Doca-treated animals injected with 0.5 ml 70° C. Ringer solution.

6. Absorption tests as under 5 with sesame oil instead of Doca.

7. Absorption tests in sesame oil-treated animals injected with 0.5 ml 70° C. Ringer solution in alternately the right and left knee joint. The unlesioned joint was used in the tests.

8. Absorption tests as under 1. Doca treated animals injected with 80° C. Ringer solution.

9. Absorption tests as under 8 on sesame oil-treated animals.

10. Perfusion experiments on animals burned as under 5 and treated with Doca as described above. Only one injured joint cavity in each animal was used and the perfusion period was 4—6 minutes at each applied perfusion pressure.

11. Same as 10 excepting that sesame oil was used instead of Doca.

12. Perfusion experiments on Doca-treated animals burned as under 8, only one injured knee joint being used in each animal. Perfusion time 4—6 minutes at each pressure level.

13. Perfusion experiments as under 12 on sesame oil-treated animals.

Sections of the joint capsules were taken from 4 animals in groups 1 and 2, respectively. Such sections were also taken from 4 animals in each of groups 5, 6, 8 and 9; these had been burned and treated with respectively Doca or sesame oil. No absorption or perfusion experiments were performed with the joints supplying these latter tissue sections. 70 minutes after the injection of the irritant the anesthetized animals were killed by intravenously injecting additional urethane. The sections were fixed in 10 per cent formalin.

## Results

From the results collected in table 9 and figs. 25, 26, 27, 28 it is evident that:

A. Pretreatment with Doca lowers the increased absorption values for both colloid and fluid in animals where the diluted culture filtrate was used as irritant (comparison between test groups 1 and 2). However, the absorption of hemoglobin in group 1 is still higher than in animals treated with only sesame oil and in which the synovial membrane was not lesioned (comparison between groups 1 and 4).

TABLE 9. In all absorption test 0.85 ml of hemoglobin solution was injected intraarticularly. The mean values from both joints in each animal = one experiment in all absorption tests except in in test group 7 where alternately only the right or left knee joint was used in each animal.

Signs: $m$ = arithmetic mean $e$ = standard error of the mean $s^2$ = variance $n$ = number of experiments $t$ : for the statistical meaning of $t$ see YULE KENDALL (1946) $p$ : the probability that the difference is caused by chance	A Hemoglobin absorbed in per cent of injected	B Intraarticular fluid volume in ml at end of test period	C Concentration of hemoglobin in g per cent at end of test period	Perfusion experiments. The experiments started 30 minutes after induction of the lesions		
				$\frac{dF}{dP}$ (ml/min/cm) $\cdot 10^5$		Breaking point in cm H <sub>2</sub> O
				Pressures be- low breaking point	Pressures above breaking point or in ex- periments not giving any breaking point	
				D	E	F
1. Doca-treated animals. Diluted culture- filtrate used as irritant	$m=60.3$ $e=\pm 1.9$ $n=14$	$m=0.574$ $e=\pm 0.024$ $n=14$	$m=3.26$ $e=\pm 0.08$ $n=14$			
2. Sesame oil treated animals. Irritant as under 1	$m=72.9$ $e=\pm 2.1$ $n=16$	$m=0.406$ $e=\pm 0.030$ $n=16$	$m=3.15$ $e=\pm 0.11$ $n=16$			
3. Doca treated animals. No injuries made	$m=51.5$ $e=\pm 1.3$ $n=13$	$m=0.596$ $e=\pm 0.022$ $n=13$	$m=3.83$ $e=\pm 0.08$ $n=13$			
4. Sesame oil treated animals. No injuries made	$m=52.9$ $e=\pm 0.9$ $n=15$	$m=0.637$ $e=\pm 0.021$ $n=15$	$m=3.49$ $e=\pm 0.08$ $n=15$			
5. Doca-treated animals. Irritant 70° C Ringer solution	$m=44.8$ $e=\pm 1.5$	$m=0.579$ $e=\pm 0.011$	$m=4.45$ $e=\pm 0.18$	$m=91^1$ $s^2=686$	$m=2489$ $s^2=16.8 \cdot 10^6$	$m=9.30$ $s^2=8.82$

6. Sesame oil treated animals. Irritant 70° C Ringer solution	$m=51.7$ $e=\pm 1.0$ $n=10$	$m=0.545$ $e=\pm 0.018$ $n=10$	$m=4.19$ $e=\pm 0.11$ $n=10$	$m=225$ $s^2=10041$ $n=6$
7. Sesame oil treated animals. Irritant 70° C Ringer solution. Only the unlesioned joint used in the tests	$m=40.2$ $e=\pm 2.9$ $n=13$	$m=0.587$ $e=\pm 0.023$ $n=13$	$m=4.76$ $e=\pm 0.15$ $n=13$	$m=440$ $s^2=42082$ $n=9$
8. Doca treated animals. Irritant 80° C Ringer solution	$m=45.1$ $s^2=97.7$ $n=9$	$m=0.625$ $s^2=0.016$ $n=9$	$m=4.19$ $s^2=0.45$ $n=9$	$m=185$ $s^2=17607$ $n=8$
9. Sesame oil treated animals. Irritant 80° C Ringer solution	$m=32.8$ $e=\pm 2.1$ $n=11$	$m=0.569$ $e=\pm 0.025$ $n=11$	$m=4.69$ $e=\pm 0.19$ $n=11$	

<sup>1</sup> Experiments in groups 5 (D, E, F), 6 E, 8 E, 9 E, were carried out on male albino rabbits. The experiments in these groups which are tested for differences between mean values were carried out simultaneously with the same test solutions.

Table groups 1—9 correspond to equally numerated text groups. (See under the heading "Experimental").

Table groups 5 D, E, F correspond to text group 10

» group 6 E corresponds to text group 11

» » 8 E » » » 12

» » 9 E » » » 13

Differences between table groups	Numerical values of differences	$t$	$p$
1 A minus 2 A	— 12.7	4.43	< 0.01
1 B » 2 B	+ 0.168	4.29	
1 A » 4 A	+ 7.4	3.58	
5 A » 6 A	— 6.9	3.94	
5 A » 4 A	— 8.1	4.90	
7 A » 4 A	— 12.7	4.40	
5 D » 6 E	— 13.4	3.18	
8 A » 9 A	+ 12.3	3.74	
4 A » 9 A	+ 20.1	9.75	
8 E » 9 E	+ 25.5	2.99	

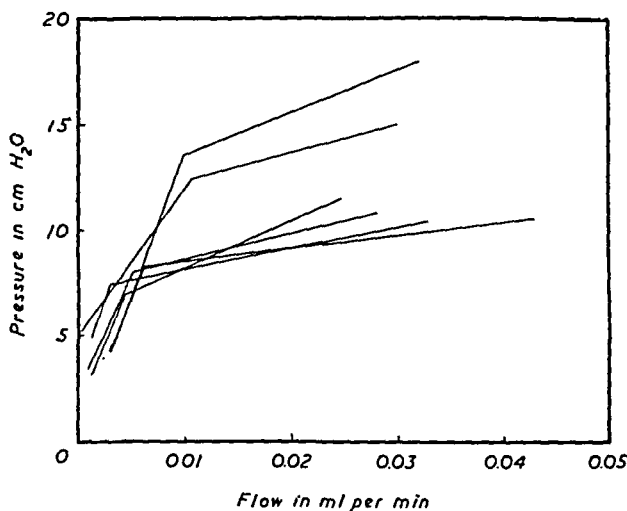


Fig. 25. Doca-treated animals, burned with 0.5 ml Ringersolution ( $70^{\circ}$ ) in the right knee joint 30' before the perfusion experiments began in the left. A breaking point is found in all experiments. Curves start at pressures where measurable inflow first could be registered and end at the highest used pressure. The two regression curves in each experiment calculated according to the method of least squares.

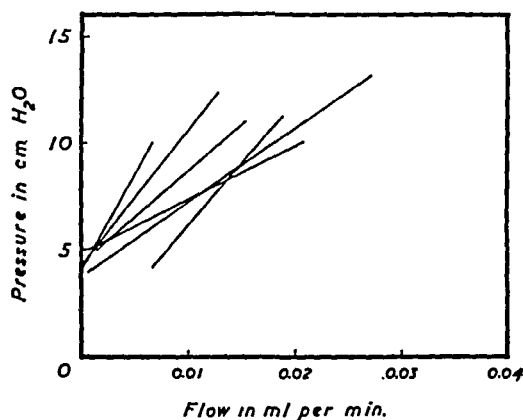


Fig. 26. Sesame oil-treated animals, burned in the right knee joint with 0.5 ml Ringer solution ( $70^{\circ}$ ) 30' before the perfusion experiments began in the left. No breaking point is found in the experiments (within the range of measurements). Curves start at pressures where measurable inflow first could be registered and end at the highest used pressure. Regression curves calculated according to the method of least squares. Flow-rates in each experiment determined at least at 7 different levels of perfusion pressure. Average correlation coefficient = 0.976. Range of correlation coefficients = 0.988—0.948 (For definition of terms and symbols see the caption of fig. 11).

B. Doca does not affect the absorption of hemoglobin and fluid from animals with intact synovial membranes (comparison between groups 3 and 4).

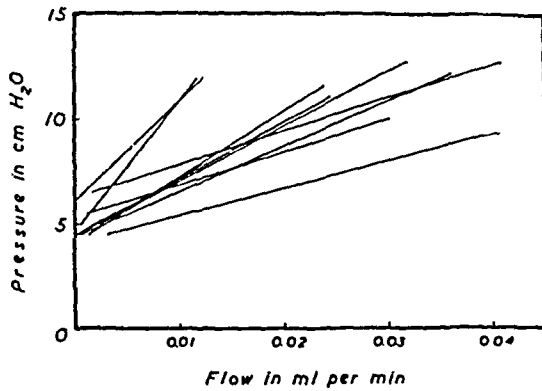


Fig. 27. Doca-treated animals, burned in the right knee joint with 0.5 ml Ringer solution (80°) 30' before the perfusion experiments began in the left. No breaking point found in the experiments. Curves start at pressures where measurable inflow first could be registered and end at the highest used pressure. Regression curves calculated according to the method of least squares. Flow-rates in each experiment determined at least at 5 different levels of perfusion pressure. Average correlation coefficient = 0.971. Range of correlation coefficients = 0.990—0.935. (For definition of terms and symbols see the caption of fig. 11.)

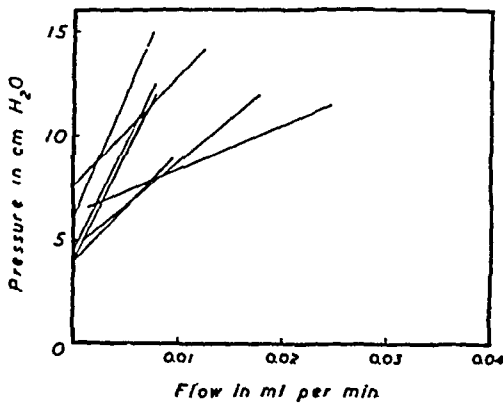


Fig. 28. Sesame oil-treated animals, burned in the right knee joint with 0.5 ml Ringer solution 30' before the perfusion experiments began in the left. No breaking point found in the experiments. Curves start at pressures where measurable inflow first could be registered and end at the highest used pressure. Regression curves calculated according to the method of least squares. Flow rates in each experiment determined at least at 5 different levels of perfusion pressure. Average correlation coefficient = 0.975. Range of correlation coefficients = 0.990—0.870. (For definition of terms and symbols see the caption of fig. 11.) Thick line in the figure indicates two coinciding regression curves.

C. Pretreatment with Doca decreases the absorption of hemoglobin from joints lesioned with 70° C. Ringer solution (comparison between groups 5 and 6). The absorption values drop to the same mean level as in group 7 wherein the absorption figures were obtained from rab-



bits with unilateral burns and the absorption experiment made on the unlesioned joint (comparison between groups 5 and 7). The absorption of hemoglobin in groups 5 and 7 are decreased in comparison to group 4. In similarly burned animals on which perfusion experiments were made, Doca increased the resistance to flow of the synovial membrane; unlike the case of animals given only sesame oil a breaking point appeared (comparison between test groups 10 and 11, figs. 25 and 26).

D. Pretreatment with Doca increases the absorption of hemoglobin in animals burned with 80° C. Ringer solution (comparison between test groups 8 and 9). In the animals given no hormone the absorption of hemoglobin is lower than in the "normal" controls (comparison between test groups 9 and 4). The resistance to flow of the synovial membrane decreased in all the animals given Doca (comparison between groups 12 and 13, figs. 27 and 28).

### Histological Examination

The technical treatment of the various preparations was carried out at the Institute of Histology, University of Uppsala, under the supervision of Doctor B. H. Persson<sup>1</sup>, who also made the histological analysis.

**STAINING OF THE SECTIONS.** The paraffin-embedded preparations were cut into 10 micron sections. Three sections from each synovial membrane were stained with hematoxylin-eosine, Weigert's fibrin stain and Mallory's phosphotungstic acid hematoxylin, respectively.

Dr B. H. Persson. "Test groups 1 and 2. Medial sections from the joint capsule, parallel to the patellar tendon and reaching to the fornix of the suprapatellar recess: slight separation of the fibrillar elements in the villi and subsynovial regions, interpreted as edema. Slight leukocytosis around the vessels, in some of the tissue spaces single threads with the histological properties of fibrin. Hyperemia in both groups, in group 2 local hemorrhages in the tissues in some of the sections". (Group 1 Doca-treated, group 2 sesame oil.)

Burned animals. Sections as above. "Test group 5. No certain histological evidence of edema or flocculation of fibrin were seen

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<sup>1</sup> I take this opportunity of thanking Dr. Persson for his valuable assistance.

in the sections, except in one synovial membrane where there was a local separation of the fibrillar elements interpreted as edema."

"Test group 6. Slight to considerable separation of the fibrillar elements interpreted as edema were seen in the villi and subsynovial tissue. All the joint capsules but one contained single threads with the histological properties of fibrin; the exception contained a local delicate meshwork looking like fibrin in one of the villi."

"Test groups 7 and 8. These groups have the same histological appearance as group 6, containing merely single threads staining as fibrin in the tissue spaces."

*Summarizing:* In the animals in which intraarticular lesions had been induced by the diluted culture filtrate histological signs of synovial edema and inflammation could be seen in both Doca and sesame oil treated groups of animals. (Groups 1 and 2.) In animals intraarticularly burned with 70° Ringer solution the histological picture exhibited signs of local edema in the synovial membrane in only one of the Doca-treated animals and in all of those given sesame oil. Both animal groups burned with 80° Ringer solution showed signs of edema.

## Discussion

PARSONS and McMASTER (1938) have found that in edema under formation the spreading of dyes through connective tissue was greatly promoted. In manifest edema the spreading was reduced. These findings are discussed on p. 55 of the present publication. McMASTER (1946 a, b) has measured the "interstitial resistance" and tissue pressure in normal and edematous, human and animal, dermal connective tissue, finding that in rapidly forming edema the tissue pressure (interstitial hydrostatic pressure of edema fluid) rises in proportion to the formation rate of edema; in slowly forming edema there is no measurable rise.

### Theoretical

Assuming that the above mentioned findings of PARSONS and McMASTER (1938) and McMASTER (1946 a) are valid for another type of connective tissue, the synovial membrane, the following chain of events might occur, if Doca decreases the permeability of blood capillaries in injured synovial membranes. A slight lesion causing but little increase in the permeability of the blood capillaries and a slight

edema in the synovial membranes should increase the absorption of hemoglobin from joint cavities (agrees with the reasoning concerning dermal connective tissue on p. 55) because the mechanical forces, the amount of pulsation energy transmitted to the tissues, ought to transport matter more effectively between slightly separated tissue elements. In such cases a reduction of the effect of the noxious agent on capillary permeability by pretreatment with Doca would lessen the increase in capillary permeability following the injury. The result of these phenomena may be a decreased absorption of hemoglobin as compared with animals not given Doca.

Rapidly forming edema in the synovial membrane causing great separation of the tissue elements would, on the other hand, tend to decrease the hemoglobin absorption in comparison to non-lesioned controls since it was assumed that in these cases the mechanical forces were not particularly active in the transport of matter (PARSONS and McMASTER). In these latter instances pretreatment with Doca, decreasing the permeability of the capillaries in the injured synovial membranes and thereby reducing the edema, may lessen the degree of separation of the tissue elements and increase the absorption of hemoglobin.

Measurements (by the methods described in chapter III) of the resistance to flow of synovial membranes may theoretically give the following results, providing that Doca really decreases the permeability of the damaged capillaries.

*In joints with slight or moderate edema the resistance to flow ought to be lessened and the breaking point abolished (McMASTER 1941 c). In very slight edema there may be a breaking point, but at each successively raised perfusion pressure level the increase in flow ought to be greater than the correspondingly increased flow in joints with non-lesioned synovial membranes. (This reasoning is analogous with McMASTER's findings (1941 c) on dermal connective tissue of mice.) In such cases the effect of pretreatment with Doca would be an increase in the resistance to flow — possibly these joints may have a breaking point.*

In groups with a rapidly developing edema of the synovial membrane things would very likely be more complicated. Measurable inflow from the perfusion apparatus (see chapter III) ought, owing to the increased tissue pressure attending rapidly forming edema (McMASTER 1946 a), to be noticeable only at higher perfusion pressures than in animals with

non-lesioned synovial membranes. Once the inflow has started, the perfusion rate at each pressure level should be a measure of the resistance to flow of the synovial membranes in animals with rapidly forming edema which resistance theoretically ought to become lower as the tissue elements separate, i. e. as the edema increases. However, in the kind of perfusion experiments performed, commencing 30 minutes after lesioning, the tissue pressure in the synovial membrane could be expected to be on the increase since a considerable edema is rapidly being formed. (This in analogy with McMASTER's findings on rapidly forming edema in connective tissue of mice; 1946 a). The effect during the perfusion experiments of this rising tissue pressure would be to make the pressure difference between the perfusion fluid and the synovial tissues smaller than could be expected from the increase in pressure in the perfusion apparatus during the experiments. (In McMASTER's experiments the pressure of the edema fluid in rapidly developing edema was rising as late as two hours after the injury.)

If this were so, the increased rates of flow at the raised perfusion pressures would be smaller than could be expected if the tissue pressure in the edematous tissue was constant during the experiments. Assuming that Doca counteracts the increase in capillary permeability caused by the injuries, the implication would be that, when an irritant causing rapidly developing edema is used, the formation rate of edema would be reduced in the hormone-treated animals as compared with untreated animals used as controls. Hence, this would retard the rate of increase of the tissue pressure in the synovial membranes. In other words, the tissue pressure of the synovial membranes would not rise so rapidly in the perfused animals as in the controls only given sesame oil. Hereby a pressure difference would be caused at each perfusion pressure between the perfusion fluid and the synovial tissues which would be greater than in the sesame oil-treated controls. Hence the measured resistance to flow in the Doca-treated animals may appear lower than in the animals given sesame oil.

**The Author's Experiments on the Effect of Doca on the Absorption of Hemoglobin Solution from Joint Cavities and on the Resistance to Flow in Synovial Membranes as Related to the Above Theories**

EXPERIMENTS IN WHICH A DILUTED STAPHYLOCOCCUS FILTRATE WAS USED AS IRRITANT. From the histologist's report on the microscopi-

cal pictures of the injured synovial membranes the following is evident. A slight edema in and inflammation of the synovial membranes exist both in the Doca-treated and control animals (groups 1 and 2 above). The absorption of hemoglobin is lower in the Doca-treated animals than in those given sesame oil, and this agrees with the above theory.

However, the bacterial filtrate contains hyaluronidase and the hormone may have behaved like a hyaluronidase inhibitor and therefore caused the measured decrease in absorption. EDLUND and JUHLIN (unpublished experiments) have established that in rabbits intramuscularly injected Doca in the doses used above does not inhibit hyaluronidase (measured as the effect of the hormone on the spreading area of intradermally injected wheals of hemoglobin solution containing hyaluronidase). According to MAYER *et al.* (1948) burning of the skin increases the amount of extractible, active hyaluronidase and, therefore, the above findings may be applicable also to the burned animals.

The increased absorption of fluid in the animals given only sesame oil (group 2 compared to group 1) may perhaps be explained by the theory verified in chapter V. In these cases hemoglobin may serve as a "carrier" for the water and the absorption of hemoglobin being increased, this leads to an augmented absorption of water and this augmentation may not be compensated by increased leakage of fluid into the joint cavity from the injured capillaries. The final result may be measured increased net. absorption of fluid during the test period.

EXPERIMENTS WITH HOT RINGER SOLUTION AS IRRITANT. The histological report makes it plain that there was edema in the groups given Doca and sesame oil and in which the temperature of the injected Ringer irritant was 80° C. In the groups burned with 70° Ringer solution the animals had edema which had not been given Doca; one of the latter, however, exhibited histological signs of local edema in one of the examined synovial membranes.

EXPERIMENTS WHERE THE IRRITANT WAS 70° RINGER SOLUTION. In these animals pretreatment with Doca lowered the absorption of hemoglobin to the level prevailing in sesame oil-treated animals burned in the joint contralateral to the tested joint. (Group 5 compared with group 7.) In this Doca-treated group the resistance to flow in the injured joints was on par with that in animals with un-

injured synovial membranes, i. e. a breaking point existed. (Group 10 fig. 25, table 9 compared with table 1, p. 15).

The fact that pretreatment with Doca causes the breaking point to persist — unlike the group of animals treated with sesame oil in which no breaking point exists and the resistance to flow is lower (group 10, table 9) — can be explained by the above theories concerning the effect of Doca on injured capillaries. Histological findings of edema, on the other hand, may explain why the absorption of hemoglobin in the sesame oil-treated animals does not exceed that in the uninjured control group (group 6 versus group 4). Depending on the degree of edema an increased or decreased transport of matter through connective tissue may occur. Therefore the absorption values may by chance be the same in sesame oil-treated animals as in normal animals with undamaged synovial membranes.

It is difficult to explain why pretreatment with Doca should lower the absorption of hemoglobin from burned joints (group 5) to the level obtained in animals given sesame oil and burned in the joint not used in the absorption tests (group 7). One synovial membrane is lesioned in this latter group of animals with unilateral burns and the reasoning and results arrived at in chapter V offer some explanation why the absorption of hemoglobin should be smaller in such animals than in unlesioned animals. But in the burned, Doca-treated animals there is no certain evidence of lesions, histological or measurable as changes in the resistance to flow, and yet the absorption is bilaterally decreased. This can only mean that in the Doca-treated animals the burning alone of both synovial membranes, despite causing no visible signs of injury, has the same effect on the absorption of hemoglobin as burning of the joint not subjected to testing (chapter V). Possibly the action of Doca may be that it is without effect on the release of the chemical products of injury (with the results suggested in chapter V) and at the same time diminishes or prevents another local response: the inflammatory reaction. The inhibiting effect of Doca on the inflammatory reaction would then be similar to the action of ethanol (PICKRELL 1938) or benzene poisoning (SCHNITZER and GODDARD 1943) on the local vascular reactions to injury.

Anyhow, since Doca is without effect on the absorption of hemoglobin from unlesioned joints (group 3 in comparison to group 4), the decreased absorption of hemoglobin in the burned animals cannot

be a direct consequence of the hormone treatment. That Doca is without effect on the absorption of hemoglobin in animals with unlesioned synovial membranes gives some support to the theory that the Doca effect on the permeability of injured synovial membranes is exerted by a regulation of the degree of edema which in its turn is due to the action of Doca on the permeability of injured capillaries.

EXPERIMENTS IN WHICH THE IRRITANT WAS 80° C. RINGER SOLUTION. The results were in agreement with the above theory that in burned Doca-treated animals the absorption of hemoglobin ought to increase as compared to animals treated with sesame oil only. (It is assumed that burning with 80° C. Ringer solution causes a more rapidly forming edema than burning with 70° C., which seems reasonable.) The alterations in the resistance to flow also followed the above theories, the resistance being decreased in Doca-treated animals. (Group 12/group 13.)

In the group of burned animals not given the hormone the absorption of hemoglobin was lower than in the unlesioned animals given sesame oil (group 9/group 4). This agrees with the above theories on the effect of pronounced edema on hemoglobin absorption. The existence of a pronounced edema in these experiments is supported by the observation that the inflow from the perfusion apparatus seemed to commence at a higher level of perfusion pressure than in animals with intact synovial membranes (figs. 27, 28 as compared with figs. 2, 3, 4, 5 p. 12, 13) in line with the above reasoning concerning the influence of raised tissue pressure on such measurements.

### Comment

Changes in the chemistry of the blood and alterations in blood pressure as the possible causes of the Doca-effects found in this chapter were not discussed, since there is no evidence saying that such phenomena would set in sufficiently soon after treatment. Nor were the possible influences of fibrin clots or intraarticular pressure during the various types of experiments discussed, because little or no fibrin was found in the histological sections (see MENKIN (1940 b) for possible effects on tissue permeability of fibrin clots) and since the intraarticular pressure was not measured.

The author has investigated the effect of Doca administered before

making intraarticular injuries on the absorption through and resistance to flow of synovial membranes. In these respects the action of Doca was such that it may confirm the opinions of previous authors that Doca decreases the permeability of injured capillaries.

Doca increased the absorption of hemoglobin from joints with supposedly severely lesioned synovial membranes, while it decreased the same absorption from more mildly injured joints. Doca was without effect on the absorption from joint cavities with intact synovial membranes. These effects were discussed in relation to theories presented earlier in the chapter.

## CHAPTER VII

### Summary

There are described original methods of measuring (in rabbit knee joints) the resistance to flow of synovial membranes, intraarticular hydrostatic pressures during absorption tests, and the amount of colloid (hemoglobin) and fluid absorbed; the validity of the methods is discussed. (Chapter III and IV.)

The measurements of the resistance to flow of the synovial membranes reveal that the author's results are partly identical to findings on dermal connective tissue of mice reported in the literature. Above a certain hydrostatic pressure, the "breaking point" (average 9 cm H<sub>2</sub>O), the resistance suddenly yields, as though the tissue elements had been separated. (Chapter III.)

The method of measuring the absorption of colloid and fluid permits the absorption tests to be carried out at varying pressures, above or below the breaking point. Measurements of the absorption of colloid and fluid reveal that — when the resistance to flow of the synovial membranes was variously reduced, e. g. by previous exercise or application of initial hydrostatic pressures above the breaking point — the absorption of colloid and fluid increased equally in animals initially subjected to "breaking" intraarticular hydrostatic pressures and in animals not so treated but preexercised. At the two pressure levels used below the breaking point there was no measurable difference in the absorption of hemoglobin solution.



This absence of pressure effects on absorption is perhaps due to the method of altering the applied pressure, viz. by different degrees of joint flexion in the various test groups. It is uncertain if the raised intraarticular pressure thereby caused really increases the pressure difference between the fluid in the joint cavity and the surrounding tissue. (Chapter IV.)

It is demonstrated that burns in one joint or previous intradermal injections of staphylococci decrease the absorption of colloid from unlesioned joints. In burned animals Ringer solution as test fluid is more rapidly absorbed from the joint not injured; the decreased hemoglobin absorption, unlike the raised Ringer absorption, can be counteracted by dibenamine. Dibenamine by itself increases the absorption of Ringer solution from joint cavities. (Chapter V.)

The author proves that the net. volume absorption of hemoglobin solution exceeds that from an equiionic solution not containing the colloid. (Chapter V.)

The effects of pretreatment of the animals with desoxycorticosterone acetate on the absorption of hemoglobin solution from injured and uninjured joints as well as on the resistance to flow of lesioned synovial membranes is studied. The hormone is ineffective on the absorption from normal joints; in injured joints it either increases or decreases the absorption and the resistance to flow, depending on the type and degree of lesions. These effects of the hormone are considered confirmative of those authors' theories who claim that it decreases the permeability of injured capillaries. (Chapter VI.)

In the theoretical sections in the various chapters the results are discussed in relation to the physiology of other connective tissue than synovial membranes; when some findings of other authors are compared with the results presented here no apparent differences can be found.

## ACKNOWLEDGEMENTS

The author gratefully acknowledges the help of grants from King Gustaf V's Jubilee Fund and the Swedish State Funds for Promoting Medical Research. Mrs. A. Käbin has given valuable technical assistance in the experimental work.

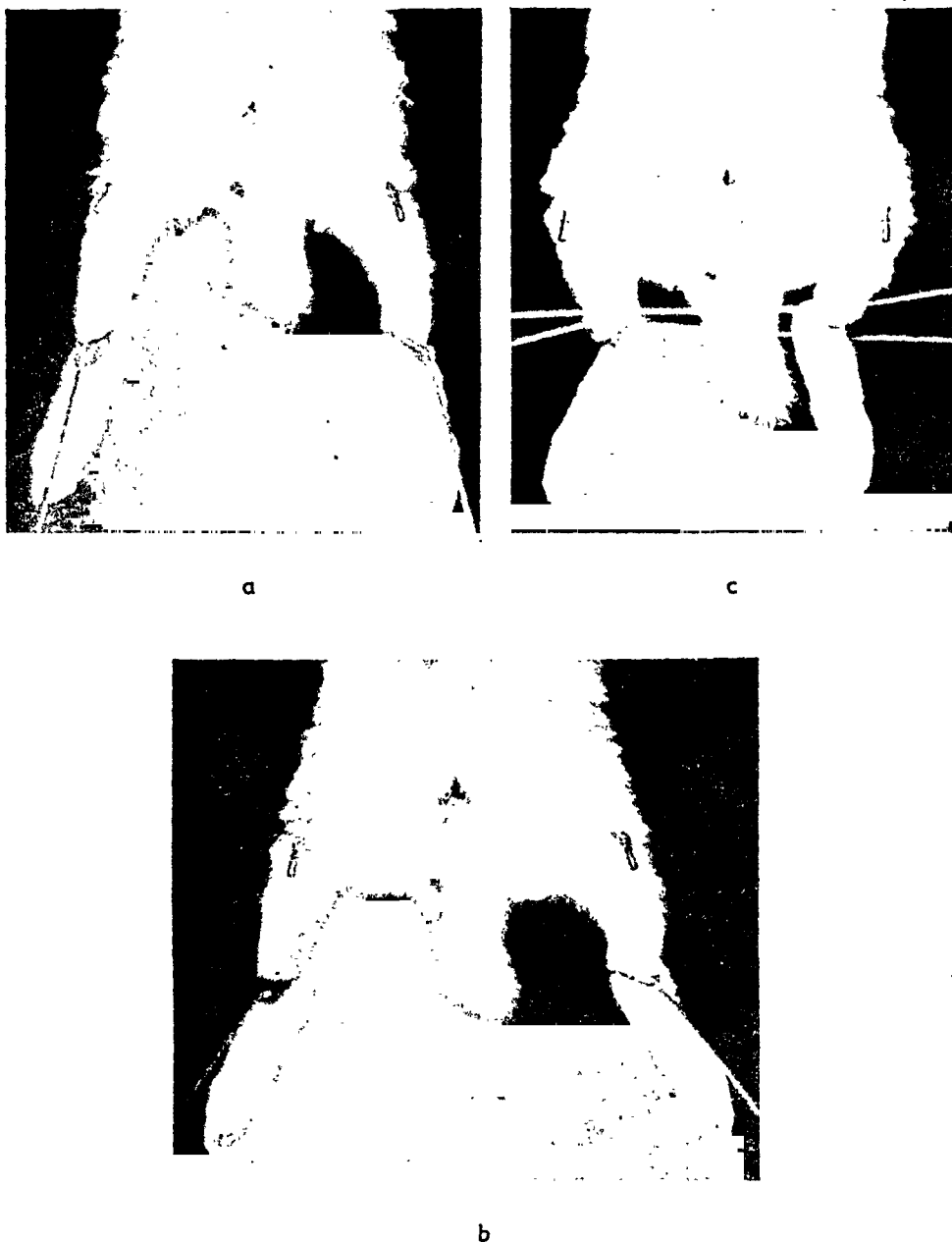


Fig. 29. a, b, c. The degrees of abduction and the manners in which the legs are tied down appear in the figs. a, 0° joint flexion. b, 60° joint flexion. c, 90° joint flexion.

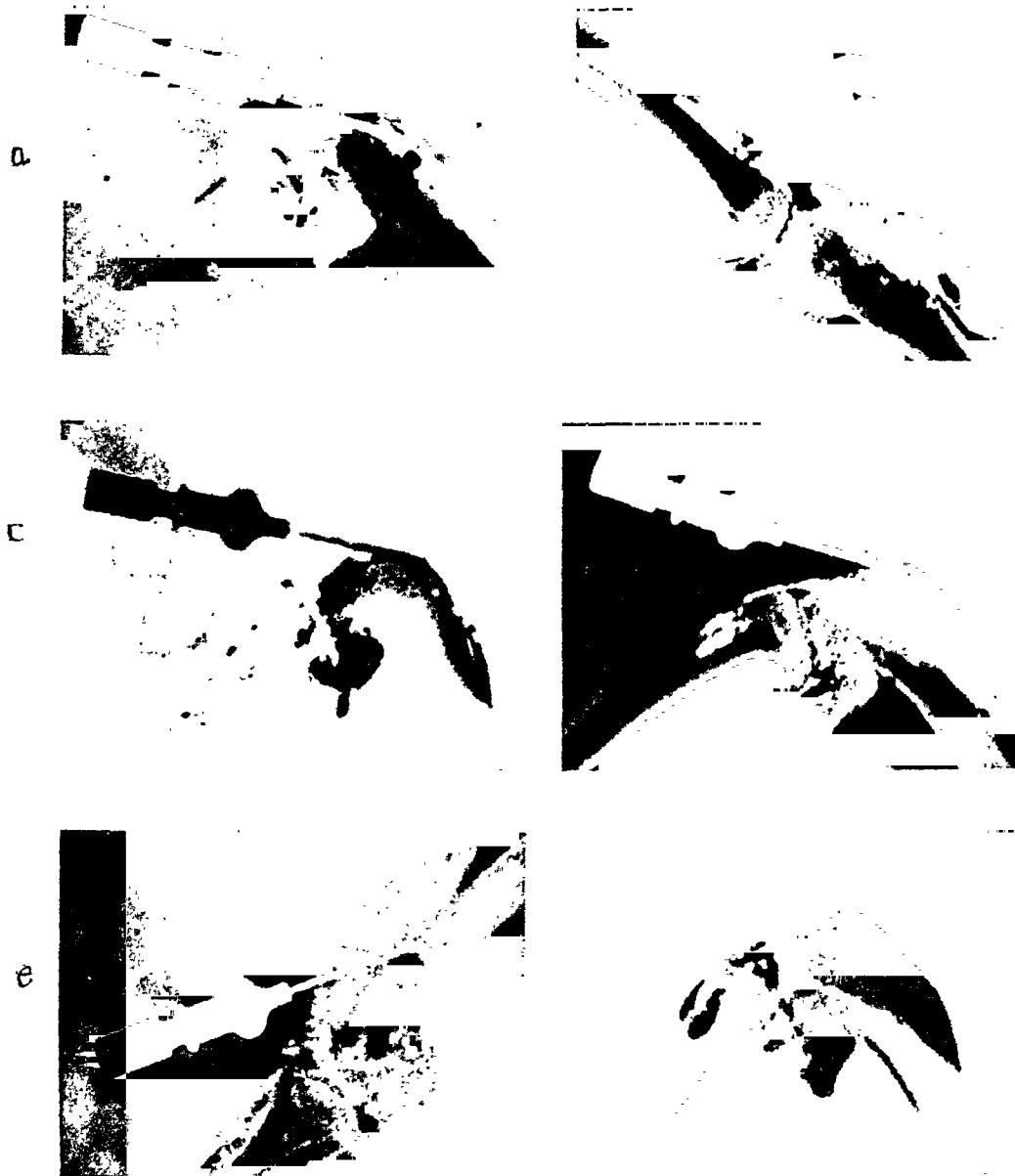


Fig. 30. a, b, c, d, e, f. Radiographs<sup>1</sup> of the left knee joint of a rabbit after injection of different amounts of lipiodol at different degrees of joint flexion. a. 60° joint flexion, 0.5 ml lipiodol. b. 0° joint flexion, 0.5 ml lipiodol. c. 90° joint flexion, 0.5 ml lipiodol. d. 60° joint flexion, 0.85 ml lipiodol. e. 0° joint flexion, 0.85 ml lipiodol. f. 90° joint flexion, 0.85 ml lipiodol. The radiographs show that injection of 0.5 ml lipiodol completely fills the joint cavity independent of the degree of joint flexion. The same events occur when 0.85 ml is injected. This indicates that the absorbing area of the synovial membrane ought to be approximately the same under the above conditions of joint flexion and intraarticular volumes.

<sup>1</sup> The author is indebted to Dr. I. Hessén of the Roentgenological Department, Akademiska sjukhuset, Uppsala, for taking the radiographs.

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FROM THE INSTITUTE OF MEDICAL CHEMISTRY,  
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STUDIES ON  
LIPIDS IN THE NERVOUS SYSTEM  
WITH SPECIAL REFERENCE TO  
QUANTITATIVE CHEMICAL DETERMINATION  
AND TOPICAL DISTRIBUTION

BY

GUNNAR BRANTE

---

UPSALA 1949





*To my Wife*



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## Preface

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Upsala, April 1949.

*Gunnar Brante.*



## Introduction

At the outset of this work several seemingly well founded micromethods had recently become available for the quantitative determination of different lipids in animal tissues. It was felt that by means of these methods it might be possible to attack many as yet unsolved problems regarding the topographical distribution of lipids in the nervous system. The fundamental question about the lipid distribution on axons and myelin sheaths is unanswered, to say nothing of finer topographical details. The all but clear situation in this field is borne out i.a. by, for example, the incomplete and controversial information in even recent textbooks and manuals. Quantitative data of the different lipids are given only rarely and then often on the basis of very early investigations with little developed methods. Thus, for example, as late as 1946. v. MURALT (127) in his discussion on the chemical components of nerves records FALK's uncertain results (cf. p. 116) from 1908 as representative.

On the grounds of such defective information, it must be difficult and perhaps misleading to draw conclusions pertaining to physiological and pathological processes in which the lipids are engaged. A careful examination of these matters seemed to be badly needed. Histochemical methods have hitherto largely failed and at best they can supply qualitative information only. Applied to appropriately selected tissue materials modern chemical micromethods should lead further.

The general plan adopted for the author's investigations was to analyze a number of structurally different parts of the central and peripheral nervous system and correlate chemical findings with known histological structure. One of my main problems, if not my chief problem, was to characterize lipid chemically the axon and the myelin sheath. In order to do so I analyzed



not only materials containing axons and myelin sheaths, respectively, in the purest form available in adult tissues, but also tried other means. A study of the lipid changes in nervous tissue during fetal and postnatal development, which in itself holds many interesting problems, was made for the same purpose, as was a study of the lipid changes during Wallerian degeneration. In order to get an idea of the part in whole tissue lipid pictures played by the supportive tissue component a series of analyses on gliomas and other brain tumour materials were carried out. The rather comprehensive material on normal tissues in different ages was collected also in order to obtain a basis for comparison in chemical studies on pathological materials. A limited number of these of special relevance in the discussion on topical lipid distribution have been included.

The investigations, therefore, were extended to cover a fairly broad field. As a consequence the analyzed material in most instances had to be too small for statistical treatment, a circumstance which in particular cases of necessity lead to some vagueness in the statements made. This admittedly is a weakness. By combining results won from different materials it was, however, not seldom possible to give clear answers on the questions for solving. An advantage of my working method was that many essential problems besides my main one could be simultaneously attacked and in some cases brought to at least a tentative solution.

It was at first believed that the inquiry into the methods would not require much work but it soon appeared that numerous methodological problems, of general nature or specific for the nervous system, had to be tackled. In fact the work of developing the methods and ascertaining their value expanded so as to make it a main part of the investigation. Nevertheless, methodologically there certainly remain many gaps to be filled and many improvements to be made.

A large number of new findings as regards the individual members in the lipid group have been published in recent years; concerning many long known facts the concepts still seem to be rather confused. Accordingly the author considered it of some importance, at least in the interest of those non-

chemists who are working within the physiology, pathology and clinics of the nervous system and for whom my results may be assumed to be valuable, to present a rather full review of the present state of knowledge concerning the composition of the lipid group. The interpretation of some of my results was hard for me, not being an expert in histology and histopathology. and may no doubt be challenged or changed, if specialists can be stimulated to contest them.



## PART I.

# Lipids in the Nervous System and Their Quantitative Determination under Various Conditions

## Chapter I.

### Lipid Constituents and Classification

For several reasons BLOOR (20) in his monography »Biochemistry of the Fatty Acids» finds the term *lipids* the most apt and convenient one for the group of fatty substances in plant and animal tissues. He defines *lipids* »as a group of naturally occurring substances consisting of the higher fatty acids, their naturally occurring compounds and substances found naturally in chemical association with them.» They are further »characterized in general by insolubility in water and solubility in 'fat solvents'.» Of necessity this definition is rather vague for the group of substances is very heterogeneous indeed and motivated in the first place for methodological reasons.

As a rule the compounds included among the lipids are composed of several molecular rests bound to each other by main valences (ester, amide, ether, acetal, salt linkages) in various combinations and releasable by different means. Since the classification of lipids is at present best based on such hydrolytically obtainable building stones in the lipid molecule a review of these logically precedes an attempt at dividing the lipids into various groups. In this review the present author will merely list such lipid substances as are recognized by recent investigators. The existence of many other lipid substances has been assumed, especially in earlier years but they have later turned out to be mixtures, partial hydrolysis products, contaminated lipids or unfindable by other investigators. They will not be discussed here.

## Constituents of Lipids

*Fatty acids*, as appears from BLOOR's definition, play an essential part in the group of substances in question. When, as in the case of a few lipids, they are not present they are replaced by related compounds (e. g. fatty aldehydes) or capable of being combined with them (cholesterol). In amount the fatty acids are without doubt the most important constituents of the lipids and to a great extent they determine the physical and chemical properties of the latter. The result of fractionation procedures which are based on solubility will, when applied to lipid mixtures, to a high degree be dependent on the fatty acids contents of the lipids. Under various names the fractions obtainable by such means formed the sets in older lipid classifications which therefore more than anything else illustrated the distribution of fatty acids in the mixtures; and, admittedly, the composition and metabolism of lipid fatty acids ought to be of great importance also in the nervous system. However, the present author has at no time intended to base his studies on fractionation of the lipids or analysis of the individual fatty acids, a most complicated procedure, difficult to interpret and more or less out of touch with target set by the author for his research. In this work the lipid fatty acids in nervous tissues will consequently be discussed only briefly.

In nervous tissue lipids the following fatty acids, all of them aliphatic, have hitherto been found: of underived, even-numbered, saturated acids those with 16-26 carbon atoms (palmitic, stearic, arachidic, behenic and lignoceric); of underived, unsaturated analogues of the former: the  $C_{18}$ -fatty acids with 1 or 2 double bonds oleic and linoleic, the  $C_{20}$ -acid with 4 double bonds arachidonic, the  $C_{22}$ -acid with 5 double bonds clupanodonic, the  $C_{24}$ -acid with 1 double bond nervonic acid; of hydroxyacids: the  $C_{24}$ -acids with one hydroxyl group, ceribronic and hydroxynervonic acid, the latter also having one double bond.

The sphingolipids excepted — in which the fatty acid is amide-like linked to an amino group — the fatty acids in the lipids are always in ester-linkage. As mentioned above there

exist lipids in which the place of two fatty acids in a polyvalent alcohol instead is taken by a *fatty aldehyde*. Stearic, palmitic and probably oleic aldehydes are those hitherto recognized in such combinations.

Alcohol radicals take a central part in most lipid molecules. The higher monovalent alcohols of importance in the nervous system are the *sterols*. Quantitatively by far the most important one is *cholesterol* which in normal nervous tissue is known to occur practically only in unesterified form. It is in itself, however, mostly classified as a lipid and then it constitutes a very considerable fraction of the lipid mixture of most nervous tissues. Other sterols too have been found in nervous tissue but only in minute quantities, e. g. dihydrocholesterol, dicholesteryl ether.

The dihydric alcohol *sphingosine* is a constituent of some lipids. Its saturated analogue, *dihydrosphingosine* has recently been found to be able to occur instead (36).

The lipid constituents described hitherto — fatty acids and aldehydes, higher alcohols and monoamino alcohols — are those which have long carbon chains and are soluble in lipid solvents and sparsely if at all in water. The other lipid products obtained by hydrolysis are preferably water soluble.

Of polyhydric alcohols *glycerol* is widely distributed in lipids. So is *galactose*. *Glucose*, on the other hand, has been detected in considerable quantities only under special conditions (82), *mannose* only in certain organisms (6). Moreover, there are signs of a *hexosamine* (possibly chondrosamine (24)) or related compound in one lipid of seemingly very complex constitution. The hexosamine is not bound as such to the lipid but in the form of *neuraminic acid*, a compound having the formula  $C_{10}H_{19}NO_9$  but of unknown structure (97). Lastly, *meso-inositol* reportedly (68) is a constituent of a few lipid substances.

Remaining known organic constituents of lipids contain nitrogen. Both *aminoethanol* (colamine), a substance long known to be lipid component, and *serine*, amino acid recently found (64, 157) in lipids, contain unsubstituted amino groups. *Choline*, which may be considered as colamine wherein the N-group has been trimethylated, is a lipid constituent occurring in most

tissues in the majority of organisms. In addition there are distinct signs of other N-compounds, probably related to the mentioned ones (37, 24). They have not as yet been identified.

Inorganic components are of importance in several classes of lipids. Among anionic compounds *phosphoric acid* radicals are most common, but *sulphuric acid* radicals also occur. *K, Na, Ca, Mg* are the cations predominantly occurring in lipids.

Thus, in lipid combination there are recognized substances generally classified as vitamins and sugars as well as characteristic protein and polysaccharide components, nitrogen bases and minerals. This suggests a metabolical connection to lipid soluble cell structures of various other classes of compounds, but at present only little is known as to the nature of such a connection.

### Combination of Constituents in Lipids

The way and sequence in which the various lipid constituents are bound to each other is more or less definitely established in several cases and will be apparent from the schematical survey below. In it the lipids are considered as generally built up about an alcoholic nucleus, to one or more of the hydroxyl groups of which the other substances have been linked. (For details see the monographies by THIERFELDER and KLENK (182), PAGE (132), and BLOOR (20), as well as the recent survey by THANNHAUSER and SCHMIDT (181) and the references in the schema).

### Lipid schema

#### I. Monovalent-alcohol lipids

General formula:  $R \cdot O \cdot X$

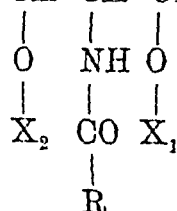
$R$  = Hydrocarbon chain or ring of certain higher alcohols, among them sterols.

<u>X</u>	<u>Lipid</u>
H	Free alcohols that in themselves may be classified as lipids e.g. cholesterol
Fatty acid* rest	Waxes, cholesteryl esters

## II. Sphingosine lipids (sphingolipids)

General formula:  $\text{CH}_3 \cdot (\text{CH}_2)_{12} \cdot \text{CH} : \text{CH} \cdot \text{CH} \cdot \text{CH} \cdot \text{CH}_2$

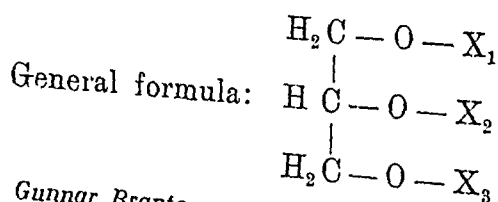
R = Hydrocarbon chain of certain fatty acids



<u>X<sub>1</sub></u>	<u>X<sub>2</sub></u>	<u>Lipid</u>
H	H	Ceramides
Choline phosphoric acid ester*	H	Sphingomyelins
		<i>Glycolipids</i>
Hexose**	H	Cerebrosides
Hexose** sulphuric acid ester	H	{ Cerebroside sulphuric acid ester (19) (Sulphatides)
Hexose**	Neuraminic acid	Gangliosides (97)

In cerebrosides dihydrosphingosine can take the place of sphingosine. The constitution of the gangliosides, which besides the hexosamine of neuraminic acid contain 3 hexose rests, is as yet little understood, the above construction being tentative and incomplete. In this connection it may be pointed out that recently cerebrosides containing 2 hexose rests per molecule have been reported (KLENK (98)).

## III. Glycerol lipids (glycerolipids)





$X_1$	$X_2$	$X_3$	Lipid
H	H	Fatty acid*	Monoglycerides
H	Fatty acid*	" "	Diglycerides
Fatty acid*	" "	" "	{Triglycerides (neutral fat)
Phosphoric acid*	" "	" "	Phosphatidic acids
Diglyceride phosphoric acid ester*	" "	Diglyceride phosphoric acid ester*	{Unit in Cardiolipins (135)
N-base <sup>o</sup> — phos- phoric acid ester*	H	Saturated fatty acid*	Lysophospholipids
Ethanolamine phosphoric acid ester*	Fatty acid*	Fatty acid*	Phosphatidyl ethanolamine (s)
" "	Fatty aldehyde***		Plasmalogens
Serine phospho- ric acid ester*	Fatty acid*	Fatty acid*	Phosphatidyl serine (s)
Choline phos- phoric acid ester*	" "	" "	Lecithins

*Glycerol lipids also containing sugars and inositol.*

This group contains some as yet incompletely analyzed substances. Perhaps the best known is a brain lipositol (66) that is regarded as a diphospho-inositide, the two phosphate radicals standing in metaposition. Glycerolipids containing P, mannose and inositol have been isolated from bacteria.

The X's in the formula under III may in each individual lipid be situated in any mutual position in addition to the one illustrated. Accordingly, the phosphoric acid radical may be linked to the central carbon atom in the glycerol molecule as well as to any of the other two. In the first case one speaks of  $\beta$ -glycerophosphoric acid, and in the second instance of  $\alpha$ -glycerophosphoric acid. Hence, there are  $\alpha$ - and  $\beta$ -types of all the glycerophosphoric acid containing lipids. If in the following nothing is said to the contrary the coexistence of these variants is presupposed. So are the variants due to the different cations and/or the different fatty acids contained. Various fatty acids occurring in different lipids will be discussed later in connection with the description of the lipids in the nervous system.

\* ester linkage  
\*\* glycoside linkage

\*\*\* acetal linkage  
<sup>o</sup> choline, ethanolamine or serine

It should be noted that several combinations that would logically complete the above schema, e.g. phosphatidic acids containing only one fatty acid, phosphatidyl monomethyl- and dimethyl-aminoethanol, and perhaps methylated serine phospholipids are missing. Actually these may never exist, but the possibility remains that they are present merely in traces, constitute rapidly metabolized intermediary stages in the known lipids, or, due to their properties, are not readily isolated in detectable amounts. In this connection considerable interest devolves upon the mentioned reports (38, 24) of signs of non-amino N-bases other than choline in brain glycerol phospholipids.

In the groups of sphingosine lipids also there logically is room for other combinations, e.g. sphingosine-galactoside, ceramide phosphoric acid ester, sphingosine phosphoric acid ester and choline sphingosine phosphoric acid diester, sphingomyelin with other N-bases than choline, esterified with a fatty acid (143) etc. Moreover, among the hydrolysis products of »protagon» there have been found (130) an ether of sphingosine with a tetradecylic alcohol. This opens up possibilities for the presence of compounds other than those in the survey. In 1946 THANN-HAUSER and SCHMIDT stated (181) »that our present classification of the sphingosine-containing constituents of brain is not yet complete.»

In addition to those in the survey a few other naturally occurring lipid soluble and related compounds having no hydroxyl groups are often classified as lipids. Among them are the hydrocarbons squalene, carotenoids, etc.

### Lipids Occurring in the Nervous System

A tissue containing all the above described lipids hardly exists, but as a rule every tissue contains several of them. Of most organisms, particularly higher, nervous tissue probably exhibits the quantitatively and qualitatively richest picture in respect of lipids. The lipids that definitely have been established to occur in nervous tissues appear in the following survey which has been so arranged as to suit the purposes of the author (see below).

The terms applied to the lipids in the tables in this chapter are those which at present occur generally in the literature. The majority are of fairly recent origin and have been formed in connection with the discovery of new fractions. An older nomenclature remains in part; sometimes, however, with a modified meaning. In order to facilitate comparison with the usage in older literature a few remarks concerning lipid nomenclature may therefore be helpful. In many older works the lecithins have designated the entire group of phosphorus containing lipids, now denoted phospholipids (or -ins, in German papers phosphatides). The phospholipids are often classified according to their N-contents.

Difference is made between monoaminophospholipids, including lecithins and cephalins, and diaminophospholipids, which are identical to sphingomyelins. Later it has been possible to divide the cephalin group (see schema) into several subdivisions, and probably the fractionation is not yet concluded. The name cephalins has formerly denoted those ether soluble brain lipids which are difficult to dissolve in alcohol (consist chiefly of phosphatidyl colamine and serine, lipositol and some plasmalogens); nowadays it usually refers to the non-choline containing phospholipids, as in the schema below. The hot alcohol releasable fraction which remains in some tissues, especially brain, after alcohol drying and ether extraction, is called »protagon», a term often seen in earlier literature. The fraction is not uniform and it chiefly consists of the sphingolipids sphingomyelin, cerebroside and gangliosides and contains hydrolecithin as well (181).

## Lipids in the Nervous System

### I. Phospholipids

#### A. Glycerophospholipids

- 1) *Choline containing = Lecithins*,  
unsaturated, containing at least one unsaturated fatty acid; saturated (hydrolecithins), containing saturated fatty acids only.  
Occurring fatty acids: palmitic, stearic, oleic and arachidonic.
- 2) *Non choline containing = Cephalins*
  - a) *Phosphatidyl ethanolamine*, containing fatty acids, more unsaturated than those of b) and no cations.
  - b) *Phosphatidyl serine*, containing the fatty acids: stearic and oleic, possibly others, and the cations: K, Na.
  - c) »*Brain diphospho-inositide*», containing fatty acids, as yet unidentified and the cations: Ca, Mg.
  - d) *Plasmalogens*, containing the fatty aldehydes: palmit- and stearylaldehyd, probably others and no cations.

#### B. Sphingophospholipids

*Sphingomyelins*, with the fatty acids: lignoceric, nervonic, stearic.

## II. Glycolipids

- a) *Cerebrosides*, per mol. containing 1 galactose rest and of fatty acids: lignoceric, cerebronic, nervonic, hydroxynervonic, n-hexacosenic, and possibly palmitic and stearic.
- b) *Cerebroside sulphuric acid* K salt = a) per mol. containing 1  $\text{KSO}_4$  rest.
- c) *Gangliosides*, per mol. containing 3(?) hexose rests (largely galactose) and 1 »neuraminic acid» rest, of fatty acids: stearic, and cations, hitherto unidentified.

## III. Sterols

- a) *Cholesterol* as free alcohol
- b)       »       , esterified with fatty acid
- c) *Dicholesteryl ether* (160)
- d) in minute quantities: *dihydrocholesterol* and others.

Considering our present knowledge of »the dynamic state of body constituents» (153) the presence, possibly in minute quantities but at any rate temporary, should be taken into account of prestages to and the respective partial hydrolysis products of the above already identified lipids.

In the author's opinion the following would be most likely: under I A 2: phosphatidic acids, possibly in the form of cardiolipins; partially methylated intermediaries between phosphatidyl colamine and serine, respectively, and lecithins, or possibly a glycerophospholipid containing an N-base entirely different from choline, colamine or serine; under I B: sphingophospholipids containing no N-base (= ceramide-phosphates), some N-base related to colamine, serine and choline, or some entirely different N-base, sphingomyelin fatty acid esters; under I B and II: compounds containing ethers of sphingosine with higher alcohols; also sphingolipids without P and glucose, e.g. ceramides, sphingosine fats (143). Normally neutral fats seem to be present in minute quantities if at all, but their presence is considered possible under pathologic conditions.

The existence in nervous tissue of other lipids or lipid constituents than those presented in the surveys is indicated by several reports (CHARGAFF et al. (38), CHARGAFF et al. (37), BRANTE (24). This will be discussed further in connection with compatible result in the present investigation.

## On the Applicability in Lipid Determination of Constituent Analysis

In order that a lipid in a material is to be considered definitely established it is at least required — as is the case as regards other kinds of substances — that the compound in question is produced in so purified a form as to be indivisible and unchangeable by continued fractionation, which does not split main valence bonds, and yields analytical values agreeing with the calculated ones. In really uniform substances the various molecular constituents as well as the elements are present in constant and definite proportions. Naturally, this also applies to lipids, but, as pointed out previously, the individual lipid groups in the above classifications as a rule include several variants which, other factors being equal, contain different fatty acids. Owing to the difference in molecular weights of the fatty acids each such lipid variant has its own definite molecular weight and its own definite proportions of constituents. The corresponding proportions for the entire group in question are dependent on the amounts in which, in the individual case, the variants are included in the group, in other words, simply on the average molecular weight. The number of variants and consequently the proportions of components in a group can be changed by physiological (e. g. age and nutrition) and pathological causes. Some idea of the theoretical limits of such shifts in the groups of nerve lipids may be obtained from the tabulation below:

		Mol. w.	% P		Mol. w.	% P
<i>Lecithins:</i>	dipalmityl	1. 752	4.12	diclupanadonyl	1. 902	3.44
<i>Cephalins:</i>	ethanolamine „	c. 692	4.48	ethanolamine „	c. 842	3.68
	serine „	c. 736	4.21	serine „	c. 886	3.50
			K „	„	c. 924	3.35
<i>Plasmalogens:</i>	palmital p.	437	7.09			
<i>Sphingomyelins:</i>	stearyl s.	749	4.15	lignoceryl s.	833	3.73
			% hexose			% hexose
<i>Cerebrosides:</i>	nervonyl c.	810	22.2	hexacosanoyl c.	840	21.4
			K „	„		
				acid ester	957	18.8

If, as nowadays often is the case, it is desirable to base a determination of the exact quantities of a lipid group in a material on a determination of a non-fatty acid constituent, more or less characteristic of the group in question, the calculation imposes knowledge of the exact constituent proportion in that group. Such knowledge may be gained by isolating the lipid group in question from the material in a purified form and evaluating the level of the constituent in question. (Cf. ARTOM (8)). Obviously one prerequisite for arriving at an exact result in such a case is that the group is isolated in its native composition. With the possibilities of fractionation available at present this is very difficult if not impossible for most lipid groups. Owing to their special fatty acids the separate variants in each group are individually soluble and since the isolation procedure as a rule includes a fractionation by solvents it is extremely difficult to avoid losses of predominantly some variants. The value obtained will therefore be merely approximately correct, but nevertheless it may be useful. Since, as already mentioned the number of variants may be changed for physiological and pathological reasons, the concentration of the desired constituent should, if maximum exactitude is to be attained, be determined in purified lipid groups from material in each one of the conditions included in the investigation. If several materials and several lipid groups are to be investigated simultaneously such a procedure implies an enormous consumption of material and time. In most cases it is more or less infeasible and often unnecessary. If the main purpose of the investigation is to compare similar materials, then absolute figures often are less important than differences. In such cases it may be satisfactory to determine the amounts of analyzed constituents in lipid groups isolated from an initial material (standard material) and then apply the figures obtained to modified materials also. According to the above table (p. 22) the inherent approximations may be considerable but probably only in very unfavourable cases. How considerable it really is in the individual case is difficult to tell. It certainly always is smaller than the maximum one, in many cases minimal, and then the method is useful. Several investigations of purified lipid fractions from nervous tissue (mostly adult whole brain) are

available. The following survey gives some representative values (the mol. weights are the minimal ones, calculated from the known P and hexose contents).

*Lecithins*: LEVENE and ROLF (111) 3.90 % P, mol. w. 795.

*Cephalins, unfractionated*: LEVENE and WEST (112) 3.87 % P, mol. w. 801; FALK (56) whole brain 3.23 % P, mol. w. 960; nerves 4.42 % P, mol. w. 701.

*Phosphatidyl ethanolamine*: FOLCH (65) and RATHMAN (142) 3.65 % P, mol. w. 849; BURMASTER (calf brain (33)) 3.34, mol. w. 928.

*Phosphatidyl serine*: FOLCH (67) 3.89 % P, mol. w. 798, as K salt (its predominant form in the tissues): 3.76 % P, mol. w. 824.

*Brain diphosphoinositide*: FOLCH (66) 7.3 % P, mol. w. 848.

*Sphingomyelins*: HACK (79) gives P analyses in brain sphingomyelins by about a dozen investigators: 3.24, 3.99, 4.10, 4.03, 4.09, 3.90, 3.96, 4.01, 3.72, 3.78, 3.89, 3.88 %, or as an average of the 11 latest results 3.94 % P, mol. w. 787.

*Cerebrosides*: Based on KLENK's data 1927 (99) for the composition of the cerebroside fraction of the brain the average mol. w. is 822, galactose 21.9 %.

[*Plasmalogens* (from muscle): FEULGEN and BERSIN (59) 6.67 % P, mol. w. 465. *Cardiolipins* (from heart): PANGBORN (135) 4.18 % P, mol. w. 742.]

While the figures for the individual phospholipids have been found to vary, the average P contents in the quantitatively more important ones lies fairly consistently just below 4 %. In probably the most works on nerve lipids the figure 25 has been used as a multiplier in computing the amount of phospholipid from lipid P values. Thus, the generally adopted factor seems to be a bit too low. By how much it will diverge from the correct figure will depend on the lipid mixture in the very part of the nervous tissue to be analysed (cf. FALK's figures above). Since it is impossible to get the absolutely correct figure the factor 25 may be used for the sake of convenient comparison with older reports. It should be kept in mind, however, that the figure is only approximatively correct. When the individual phospholipids have been determined separately and calculated by means of own factors, the total phospholipid value should be corrected as required.

Reporting in micro-equivalents per weight unit of tissue has many advantages but does not permit direct comparison with the commonly used method of expression in weight %.

### Summary

*A survey is presented over the present state of our knowledge of the chemical nature and constituents of lipids known to be present in nerve tissues. The essential requirements for and the applicability of the increasingly employed principle for quantitative lipid analysis by determining characteristic lipid constituents are dealt with in detail.*



## Chapter II.

### Planning and Developing Methods for Lipid Determination in Small Samples of Nervous Tissue

For purposes defined in the introduction the author using as small samples as possible of fresh nervous tissues wished to develop as simple a method as possible of producing as complete and uncontaminated lipid extracts as possible. These would then be subjected to various microdeterminations of the characteristic lipid constituents. Knowing the amounts in which these constituents are contained in their respective lipids, the quantities of the latter could be approximately computed from the results of the analysis.

The lipid constituents, on which a study like the one outlined could be based appear in the above table (p. 16). As mentioned, due to their complexity the fatty acids were not intended to be studied. When the experimental work was commenced no convenient micromethods seemed to be available for the determination of acetal phospholipids or sphingosine. Separately, in collaboration with SVENNERHOLM (28), the physiological variations of »neuraminic acid» was studied. For other lipid constituents several suitable micromethods were available and on them are based the author's investigations. The procedure is described in detail in the appendix on methods.

As the used procedures largely are adaptations of recent methods already published elsewhere by their inventors, it may seem unnecessary to recapitulate them. However, the original papers are widely disseminated in numerous issues of several journals and therefore it should be convenient for the reader to have them collected in one place for easy reference, should he find them interesting or conducive to further experimentation. Moreover, on several points they have been rationalized

in addition to being presented in a uniform, practical, directly accessible form.

In this chapter will merely be discussed the reasons why this very combination of methods was adopted, its advantages and disadvantages, as well as some qualitative investigations into various isolated brain lipids.

### Subdivision of Tissue to Be Extracted

For the purpose at hand an ideal subdivision would entail that *the lipids in the tissues should be rendered fully accessible to the subsequent extraction*. Each cell, each nerve fiber should be accessible to the extraction liquid. Undivided portions or the formation of lumps must be avoided as their centres can become very inaccessible and difficult to extract. Subdivision in grinders or mortars — a method adopted by many authors. — is hardly satisfactory when applied to small tissue materials (cf. BACKLIN (10)). Moreover, pounding in mortars as a rule includes an admixture with sand, which, although in itself indifferent, should be avoided as any »extras» may be troublesome, e. g. in connection with weighing. The methods appearing satisfactory to the author were alternatively grinding in a microgrinder (193) or sectioning on a freezing microtome. Since in earlier studies (25) the author already had had good experiences of the latter method, it was chosen for this work. If the sections are cut extremely thin an ideal subdivision would theoretically be obtained. But very thin sections are liable to involve technical difficulties and when the cutting is done manually the time lost is excessive. A compromise must therefore be made by choosing a thickness which is sufficiently effective and at the same time practical. The author has used the thickness 7.5  $\mu$ ; as far as possible the cuts were made at right angles to the fibres and faulty sections and lump formation were avoided.

The suspension of tissues obtained by shaking so treated material in the extraction liquids is very fine and homogeneous. Its superiority over the mortar method was clearly demonstrated in experiments.

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Though otherwise adequate the microtome technique is time consuming. When a really effective microblender is available it is probably to be preferred. Subdivision with a microblender could suitably be performed in the extraction medium. Ultrasonic subdivision in the extraction liquid is a possibility that should be attempted and which perhaps might prove superior to the other methods: it should also be feasible with freeze-dried tissues.

## Extraction

The *requirements* initially put on the extraction procedure were: *applicability to fresh tissues, completeness and selectiveness as regards lipids and simplicity of technique so as to be suitable for routine use.*

Since drying of unfrozen tissues by experience entails a risk of reduced releasability and no freezing drier was available, the author considered it necessary to extract the tissues in a moist state. For most of the more selective solvents this implies that some sort of **dehydration**, must precede the extraction, which may be carried out with water-miscible lipid solvents such as acetone or alcohol. These solvents also cause a denaturing of proteins and a setting free of protein bound lipids. As a matter of fact hot ethanol or methanol in excess is capable of quantitatively releasing most lipids from tissue, particularly when they are present in mixtures and mutually increase their solubility. But alcohol also releases a troublesome quantity of non-lipid tissue constituents. Although in certain respects convenient, alcohol extraction is therefore unsatisfactory without special precautions. The power mentioned of alcohol to release lipids from their combinations with other substances in tissues does, however, in most cases render alcohol treatment desirable or necessary. Using alcohol the object will then be to prevent contamination by non-lipids.

Several means have been attempted to realize this object:

- a) admixture to the alcohol of a certain proportion of more selective lipid solvents (which in addition may increase the power of alcohol to dissolve certain lipids).
- b) pretreatment of the tissue so that troublesome water soluble constituents are removed without loss of lipids.
- c) ridding an evaporated alcohol extract from non-lipid im-

purities, usually by reextraction with a more selective lipid solvent.

A 3:1 alcohol-ether mixture (according to BLOOR (20)) is much used for lipid extraction. On some materials the mixture has proved adequately selective, e. g. in respect of phospholipids (50). The author had previously used this method in studying plasmalipids (26) and lipids in rat tissues (25). Among other things these studies showed (unpublished) that an alcohol-ether extract of rat liver contained a very small but measurable quantity of phosphorus which could not be reextracted with benzene. Results (extinction values):

Material	P h o s p h o r u s		C h o l i n e	
	in alcohol-ether extr.	in $C_6H_6$ re-extract of the alcohol-ether extract	in alcohol-ether extr.	in $C_6H_6$ re-extract of the alcohol-ether extract
Liver a	0.501	0.495	1.022	1.036
" b	0.429	0.419		
" c	0.392	0.380		
" d	0.456	0.444		

Evidently, no choline phospholipids were lost in the reextraction. As cephalins throughout are soluble in benzene the differences in the P-values are probably due to non-lipid phosphorus.

Additional experiments showed that similar contamination occurs in alcohol-ether extracts of nervous tissues. The total phosphorus value in an alcohol-ether extract of whole brain was approximately 4.5 % higher than its value for an equivalent quantity of its reextract in hot chloroform. On the other hand the total choline value was the same in both cases. The phospholipids are all soluble in chloroform. Probably the evaporation and reextraction procedure used does not in itself entail phospholipid losses (see below). From all this it appears that the extra quantity of phosphorus in the alcohol-ether extract probably is of non-lipid nature.

Besides, it is simple to demonstrate that the primary alcohol-ether extract of fresh nervous tissues contains water soluble impurities. After completed reextraction with chloroform of

an evaporated alcohol-ether extract there is left on the walls of the vessel a considerable, usually white, fine-grained residue. This substance very easily dissolves in distilled water, has a salty taste and among other things contains phosphorus. The alcohol-ether extract of tissues fixed in formalin contains little or no such residue, which fact further supports the assumption that it mainly consists of water soluble impurities. It probably is equivalent to the »carnithin» described by MACLEAN (117). In lipid extraction this is obtained in the alcohol fraction, contains a little P, purines, amino acids, sugars, creatinine etc. and has been found little soluble in chloroform (28).

An increase of the ether proportion to 2 parts to 3 of alcohol did not change the results. Possibly the extraction of lipids was rendered somewhat less complete.

The results of analyses carried out on one and the same nervous material but with different extraction procedures were (expressed in mg P per g dry substance): alcohol-ether 3:2 + chloroform reextraction, 9.72; alcohol-ether 3:1 + chloroform reextraction, 10.0; alcohol-ether 3:1, 10.16.

Other mixtures than the ones mentioned were not tested. However, it is obvious that *an admixture of ether according to BLOOR is not enough to prevent troublesome impurities in the alcohol extract.*

**Pretreatment of the tissues** to remove contaminations can be variously carried out. For example, the lipids can be precipitated together with the proteins by an aqueous solution of trichloroacetic acid, colloidal iron or formalin. The author has not tested the treatment with *trichloroacetic acid* but it would seem to be *a priori* unsuitable for organs rich in acetal phospholipids, for these lipids are easily broken down in an acid environment. The effect of acid treatment on the mineral contents in the lipids evidently also implies alteration of the solubility of some lipids (67).

Pretreatment with *colloidal iron* is according to FOLCH and VAN SLYKE (69) a satisfactory method of avoiding certain contaminations in a subsequent alcohol-ether extraction of plasma. The author tested this method on finely divided nervous tissues suspended in physiologic saline so that the concentration of

dry substance would correspond to that in plasma. However, it turned out that with this material considerable amounts of lipids were lost. (SPERRY reports similar experiences (164). For results see Table I. It may be noticed that some lipids (e. g. cholesterol, lecithins, cerebrosides in white matter) are less affected than others. The precipitability may be dependent on the degree of acid dissociation of the lipid.

At one time SMITH and MAIR (162) used *formalin* treatment of the tissues before extracting the lipids. Later several authors have found that formalin has a destructive effect on some lipids. The present author has also extensively studied this formalin effect, particularly with respect to the usefulness of pathological material fixed in formalin for studies on the nature of lipids. These experiments are described in detail in chapter V. Here it will merely be emphasized that formalin in the long run destroys certain kinds of lipids, above all those belonging to the phospholipid group. Brief treatment with formalin in a physiologic salt solution could, however, perhaps be developed so that it becomes suitable for the removal of water soluble contaminants. The author has not gone into this question.

Lastly, *dialysis* at a low temperature is a conceivable method of getting rid of water soluble substances. The author has used this method in connection with autolysis experiments. As a routine method it is, however, far too time consuming and technically complicated. Moreover, it offers particular difficulties in dehydrating the extract and in time entails the formation of decomposition products. The author has consequently adopted this procedure in special cases only.

If therefore the various, hitherto available, methods of pretreatment for lipid extraction all suffer from several disadvantages, it must be admitted that so far we do not possess an ideal reextraction agent. The most lipid selective ones (petroleum ether, ether) are often more or less inferior solvents for some lipids. Those that dissolve all lipids often dissolve impurities too. Some are uncomfortably poisonous (benzene), others (petroleum ether) far too volatile for convenient and certain apportioning, again others are difficult and time consuming to evaporate (alcohols).

Already at an early stage the author arrived at the conclusion that *chloroform* ought to be a suitable reextraction agent. Hot chloroform dissolves all hitherto known kinds of lipids in brain tissue (perhaps fairly badly the gangliosides), dissolves slightly if at all most other substances known to occur in such tissue and it finally is convenient to work with (boils without bumping, easy to pipette) and not too toxic. It is of course a disadvantage that chloroform on evaporation has a tendency to »creep» along the walls of the vessel and that in so doing it brings some lipid matter along. This phenomenon can, however, be counteracted by blowing away the fumes with  $\text{CO}_2$  during the evaporation. But all the same a source of error due to co-solution of some non-lipid substances remains. Probably this influences the results, especially in determining total lipid contents and some nitrogenous substances (see below). ARTOM (8) has used a lipid extraction procedure similar to that of the author. He found it highly effective, leaving only traces of fatty acids in the extraction residue. However, he also suspects some slight contamination. As regards brain ARTOM's findings have been confirmed very recently (124). *Correctly, a chloroform reextract therefore should be rinsed.*

In determining cerebroside in blood and brain BRÜCKNER (29, 31) washes a chloroform reextract with trichloroacetic acid. The present author has to some extent studied the usefulness of this method; after the treatment the chloroform extract becomes very cloudy and therefore less suitable for subdivision. Moreover, the acidification as usual entails the risk of decomposition (e. g. of acetal phospholipids). On the other hand washing of the chloroform extract with distilled water should result in a solution of some phospholipids in the water phase. Under such circumstances a dialysis procedure would be preferable. But even at this stage the latter method suffers from some of the previously mentioned disadvantages, and the author has not tried it other than in purifying cephalins according to FOLCH (65) on a macro-scale. In quantitative analysis on a micro-scale considerable difficulties would certainly be encountered.

Therefore, *despite the risk of slight contamination the*

author has routinely used a primary extraction with alcohol-ether, 3:1, followed by a reextraction with hot chloroform which also was used to release any lipids remaining in the tissue residue after the alcohol-ether extraction. By this means an absolutely clear solution is obtained already at the outset — even after cooling to room temperature — but in which for some tissues (white matter) a white flocculation generally separates out at the surface after hours or days. This flocculation probably consists of lipids belonging to the protagon fraction. At least in the beginning it usually easily redissolves on heating, but as a rule the extract should as soon as possible be allotted to the various determinations.

The question of the effectiveness of the adopted extraction technique deserves some consideration. Firstly it should be pointed out that the author has used at least as large a portion of alcohol (75 ml) as prescribed by BLOOR per g fresh tissue, as a rule considerably more. By means of control experiments it was proved that a tripling of the quantity did not influence the result.

It has been stressed that, due to the risk of decomposition lipid extraction with hot alcohol should not be too prolonged. It has simultaneously been emphasized that boiling for some time in alcohol is necessary for complete extraction. For example, BACKLIN (10) obtained a maximum yield in his experiments by boiling in alcohol for 1 hour. The present author has compared the results of alcohol-ether extraction during 1 and 4 hours and could find no difference in yield.

It has long been known that owing to the ease with which certain lipid fatty acids oxidize with consequent change in solubility of the lipids and to the tendency to decompose of some lipids certain *precautionary steps* are required when lipid extracts are to be evaporated; for example, low temperature, diminished pressure and an indifferent atmosphere, e. g. CO<sub>2</sub> or N<sub>2</sub> (20). Since the latter measure would involve technical difficulties unsuitable in routine work, its necessity was tested. Aliquots of alcohol-ether extract of nervous tissue were evaporated to dryness with and without leading in of CO<sub>2</sub>, in both cases at temperatures below 60° at the beginning and below 50° at the end of the evaporation and under



maximum vacuum obtainable by water suction. Both samples were similarly reextracted in alcohol and the phosphorus contents determined. The values were practically the same (respectively 580 and 589). Hence, at least for phospholipids (which ought to be most sensitive), it appeared that no disadvantage accrued from the exclusion of the CO<sub>2</sub> current. In routine work, therefore, the author in order to protect the lipids restricted himself to low temperature, reduced pressure and short drying times.

In testing the power of several different extraction liquids of releasing choline phospholipids from tissues ENGEL (52) found that methanol was most effective. Among the less effective was an alcohol-ether mixture in the proportion 1:1. The present author has carried out experiments wherein aliquots of material were subjected either to extraction according to ENGEL with methanol for 8 hours or alcohol-ether 3:1 for 2 hours. The choline concentration in the extracts was determined. Results:

Extraction with	concentration, in %, of choline phospholipids	
	from cattle liver	from cattle spinal marrow
Methanol	2.65	3.69
Alcohol-ether 3:1	2.62	3.88

But it was mentioned above that choline lipids in an alcohol-ether extract had proved completely reextractible with chloroform. Therefore, *as far as choline phospholipids in the tissues are concerned, the author's method of extracting with alcohol-ether followed by reextraction with chloroform is probably not inferior to any other method known.*

Finally, the author in many cases studied *the possibility of extracting additional lipids* by boiling for 6-8 hours each with methanol and then chloroform of the residue after routine primary extraction. As a rule the resulting extracts were cloudy. Cholesterol or cerebrosides could not be demonstrated in them,

and choline was present in negligible quantities if at all. On the contrary they contained an evaporation residue that sometimes could amount to 3-6 % of the total lipid value in fresh tissue and up to 8 % of formalin fixed tissue. The residue was to a great extent composed of phosphoric acid compounds — converted into phospholipid the total phosphorus could amount to between 2 and 10 % of the total phospholipid value in the tissue in question. Approximately 90 % of this phosphorus was rendered acid soluble by treatment with KOH. Possibly, therefore, it may be a cephalin like substance which is fairly strongly bound in the tissue. However, ARTOM's results, mentioned above, speaks against this. The residue may also largely be composed of non-lipids which are released during the methanol treatment and pass through the filter paper in finely suspended form. The opacity of the filtrates corroborates this view. Owing to the uniformity with which the method adopted by the author was performed this additional extractible fraction obviously had little influence on results to be compared (see e. g. p. 84).

The possible occurrence of non-lipid contaminants in the final chloroform extracts have also been studied. The contents of inorganic or organic extractives, which are those substances to be most expected as contaminants, is in grey and white matter about 1 % each. As regards compounds known to be or to contain substances that also occur bound in lipids, the approximate amounts in extractive form in the brain have been reported (cf. e. g. PAGE (132)) as follows (in % wet weight): free hexose 0.03-0.05; hexose monophosphoric acid ester (as hexose) 0.05-0.06; glycogen 0.07-0.18; inositol 0.3 (198); free amino acid-N 0.10 (133); ethanolamine 0 (128); choline 0.015 (114); phosphorus in inorganic form 0.06, in organic non-lipid non-protein form 0.01-0.05; sulphur in inorganic form 0.003, neutral sulphur 0.010-0.020; potassium 0.036; sodium 0.020; calcium 0.0015; magnesium 0.0025.

In addition to the above substances there must be present different intermediary products formed in the synthesis or decomposition of the lipids.

Thus, it is evidently a matter of quite considerable quantities. In relation to the lipids the total extractives in grey matter

amount to approx. 25-30 %, in white 5-10 %. Non-lipid hexoses amount to 1.5 times the cerebroside hexose; free inositol 3 times, total non-lipid inositol 5 times the brain diphosphoinositide inositol; total free amino acids 3 times the lipid amino acid; free choline 5-10 % of lipid choline in grey matter, 2 % in white; total extractive phosphorus 75-90 % of lipid P in grey matter, 30 % in white. Glycerophosphoric acid is assumed to be present in free form only as split product of phospholipids (84).

It should be noted that the figures are only approximately correct and refer to average conditions in adult normal nervous tissue. Although conditions may be very different, the figures still give some idea of the *maximum possible error that may be caused by the cosolution of these substances. For ethanolamine and choline it evidently is small, for other lipid constituents large.*

In an experiment where certain lipid constituents (choline, glycerol, glycerophosphate, galactose, inositol) in free, water soluble form and large quantities (5-20 mg.) were added to subdivided white matter (600 mg.) from spinal marrow, and the mixture was extracted in the usual way the result was a homogenously opaque chloroform extract. On analysis it was found to contain considerable amounts of all the additions. While this experiment undoubtedly showed that the compounds in question were coextractible, it is not certain that they are so when they are included in cell structures. As usual the chloroform reextract of the nervous tissue alone was absolutely clear.

In analyzing chloroform reextracts from various tissues and under diverse conditions contamination was in many cases probable. Already the colour (e. g. yellowish in extracts from jaundiced or hemorrhagic material) sometimes suggested that. The sum of the analytically determined lipids in the extracts was mostly considerably lower than expected from the total lipid determination, as a rule 10-12 %.

In paper chromatography (24) of hydrolyzed chloroform reextracts spots sometimes developed — particularly spots indicatable by ninhydrin — which did not appear if the extracts had been dialyzed against water before the hydrolysis. At least for grey matter the result of amino acid determinations plus

colamine determinations gave values so high as to be practically unreasonable. Consequently some amino acids can contaminate the chloroform reextract. Apparently free hexose can occur in minute quantities in extracts of grey matter (observable in the nonhydrolyzed samples in the cerebroside determination), but extracts of white matter as a rule contain practically no hexose. A water extractible form of inositol possibly occurs to some extent in the chloroform reextract (see p. 98).

If some non-lipid contaminants undoubtedly can occur, it is equally certain that others do not. The dialysis experiment (p. 88) and the formalin experiments (p. 98), for example, prove that the choline and phosphorus values are but little reduced by the treatments (for moderately long times) which would have been the case had they partly been composed of water soluble impurities. The same experiments likewise show that the above mentioned excess (unidentified fraction) of extractible solids in relation to analyzed lipids only partly disappears when the water soluble impurities are removed. If despite this the excess is due to non-lipid impurities these must mainly come from nondialyzable or water insoluble non-lipid substances in the tissues. *In the author's opinion, however, a great part of the unidentified fraction rather is due to the presence of unanalyzed lipids and the fact that the factors used in computing the quantities of the individual lipids from the values of their characteristic constituents are only approximately correct and with regard to the phospholipids probably too low.*

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Although the adopted extraction technique is not ideal and under unfavourable circumstances theoretically might entail the presence of disturbing non-lipid contaminants, practical experiences apparently go to show that it as a rule is satisfactory. In the separate instances due regard must, however, often be taken of a possible source of error arising from the mode of extraction used, but this will be more fully discussed in the part dealing with the analysis of tissues.

### Dry Substance Determination

Most simply the determination of dry substance may be made in immediate connection with the weighing of the samples to be extracted. If the finely subdivided tis-

sues are weighed in a preweighed receptacle, carefully mixed and a sample to be extracted removed, the rest can be dried and weighed. Thus will be obtained the contents of dry substance in exactly the same material as is extracted.

The problem involved by this determination is the method of carrying out the drying. Different means are available. The choice stands between *vacuum dehydration* at relatively low temperatures and *air drying* in an oven and some combination of both methods. Without special precautions vacuum dehydration alone is time consuming and involves the risk of incompleteness, heat drying can cause oxidation, evaporation of other substances than water etc. (cf. TEAGUE 178). The present author chose brief drying at an elevated temperature of spread out, thin sections of the material. All samples were consistently dried in the same manner.

By repeated control tests the *completeness* was checked. A prolongation of the drying time from 2 to 24 hours did not result in a significant loss of weight. Exsiccator drying after the heat drying did not influence the result. Exsiccator drying alone over  $H_2SO_4$  with the aid of suction to constant weight (48 hours) in comparison to the routine drying method showed a little higher values for dry substance (in two different materials, both from white matters, the difference was 0.4 % resp. 1.8 %).

*Error.* This has not been estimated separately. Its order, however, seems to be in the vicinity of that for total lipid determination (see below), i. e. a few tenths of a mg. A few double determinations were done; examples: 36.1-36.2; 28.5-28.5; 29.0 (undivided sample) -28.4; 32.5 (undivided) -32.5. The amount of dry substance for weighing was seldom below 30 mg. Thus the error of a single determination ought to be about  $\pm 1$  %.

### Total Lipid Determination

It was considered valuable to have an idea of the total quantity of substance contained in the chloroform extract. It could, for example, supply information as to whether the tissue contained significant quantities of other lipids than

those determined in separate analyses. Moreover, total lipids are easy to determine by simply evaporating and weighing an aliquot of the chloroform extract. Provided the evaporation was gently carried out the weighed substance could later be useful for some other determination. The drying technique adopted by the author proved satisfactorily mild and still highly effective. Continued drying never had any effect on the result.

The value obtained is called *Total Lipids*. Manifestly these must also include any contaminants present which, however, according to the above discussion probably are of minor importance.

*Error.* The standard error of a single determination (N. B. includes errors of pipetting, evaporation and weighing) was found to be  $\pm 0.18$  mg for quantities of 10-30 mg, i. e. at most  $\pm 1.8$  % (based on 10 separate double determinations).

### Total Phosphorus Determination

In studying lipids the lipid phosphorus generally and suitably is determined by means of a colorimetric method for inorganic phosphorus following oxidation of the substance. The latter is often carried out with for example perchloric acid (KING (96), SPERRY (165)), or sulphuric acid followed by 30 %  $\text{H}_2\text{O}_2$ . A colour, in strength proportionate to the quantity of phosphorus and useful in microdeterminations, can be caused by treatment with molybdate in acid solution and subsequent reduction of the formed phosphomolybdic acid with e.g. aminonaphtholsulphonic acid (FISKE and SUBBAROW), »amidol» (51), or stannous chloride (108).

Already in earlier works (25, 26) the present author has with advantage adopted a modification by TEORELL (179) of FISKE-SUBBAROW's (62) determination, including sulphuric acid-30 %  $\text{H}_2\text{O}_2$  oxidation and aminonaphtholsulphonic acid reaction. This method is not one satisfactory for the very smallest samples but it has turned out to be adequately sensitive, easy to use and reliable in the hands of the author.

Some possibly *sources of error* in the method have been studied.

A certain concentration, not too strong and not too weak, of the sulphuric acid is required for the development of the colour (see STEWART and HENDRY (171)). Since in the author's method the sulphuric acid is present already during the oxidation a check was wanted that too much does not vanish or become used up. TEORELL (179) puts the lower limit of final sulphuric acid at 0.33 ml per sample, i. e. 1.32 % concentration in solution for colorimetry. Experiments were carried out in which increasing quantities of neutral fat were oxidized in sulphuric acid followed by addition of a given quantity of phosphorus standard to the liquids. Results:

Series I			Series II			
No.	Addition of fat mg.	Ext. Coeff.	No.	Addition of fat mg.	Vol conc. $H_2SO_4$ ml.	Ext. Coeff.
1	6.0	219	1	17	0.5	525
2	8.5	228	2	17	0.5	539
3	14.5	211	3	17	0.65	535
4	21.8	217	4	17	0.65	535
5	0	217	5	0	0.5	529
			6	0	0.65	529

The results prove that *any unfavourable action of the total lipid quantity*, which never exceeded 5 mg in my experiments, *can be excluded*. The quantity of  $H_2SO_4$  added to the samples can without risk be increased to 0.65 ml.

Furthermore, it is generally accepted in the literature that phosphorus analysis for phospholipid determination in lipid materials by methods similar to the present author's satisfies all reasonable criteria (cf. GORTNER (74), SPERRY (165)).

*Standardizing.* The standard tests run parallel with every experimental series consistently gave a rectilinear curve passing through or close to *origo*.

*Error.* Determinations were consistently duplicated (on two different quantities of the same chloroform extract). On 50 double determinations with a mean amount 0.026 mg P per sample the standard error of the single determination was found to be  $\pm 0.0011$  mg P, i. e.  $\pm 4\%$ . The error for a double determination must lie considerably lower.

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Still smaller quantities of the substance would be required by a modification like SPERRY's (165) which accordingly would be preferable when the material must be sparingly used.

### Determination of "KOH decomposable" Phospholipids

A method reportedly satisfactory for the determination of monoaminophospholipids and based on a selective liberation and solution of the phosphorus in these lipids by means of a mild alkali and acid treatment has been described by SCHMIDT et al (148) and adopted by the present author. Largely the procedure in the original work was followed. However, the solution of the lipids in alcohol before the KOH hydrolysis was considered unnecessary and was therefore left out (HACK (78) apparently also excluded this step). Moreover, a 12 % solution of trichloroacetic acid was used in place of a 10 % one for the precipitation.

It could be experimentally demonstrated that inorganic phosphorus or glycerophosphate that had been subjected to the entire procedure (including hydrolysis) was recovered to 98-100 %. The monoaminophospholipid phosphorus value was the same in an extract made up in the usual manner from white matter of spinal cord of adult cow for KOH concentrations between 0.7 N and 2 N, at temperatures from 34-45° and with hydrolysis during 24-48 hours, the latter holds also for formalin-treated material. Consequently small variations from the norms given in the Appendix on Methods do not significantly influence the result. Solving in 0.2 ml ethanol before the addition of KOH did not change the result on white matter, nor did adding extra HCl to the hydrolysate to 0.1 N concentration before trichloroacetic acid addition (used by HACK (78)).

The *selectivity* of the methods has been investigated by SCHMIDT et al. (148) and later by HACK (78). In so doing they used purified specimens of lecithin from eggs, heart and brain, cephalin mixtures from brain (also human), lipositol (raw material not stated), acetalphospholipids from brain, sphingomyelins from lungs and brain. In the purification all forms of alkali treatment were avoided. In all these materials, separate or in mixtures, both investigators demonstrated the



lecithin and cephalin phosphorus to be practically 100 % determinable, while the sphingomyelin phosphorus was altogether excluded from the determination.

If an extract contains only the ordinary lipids the method should, therefore, tell the contents of all phospholipids except sphingomyelin phosphorus, i. e. the sphingomyelin phosphorus would constitute the difference between total phosphorus and »KOH-releasable phosphorus». Such seemed to be the case in using the method on extracts of some nervous tissues (particularly grey), while others (particularly those rich in myelin) exhibited discrepancies from results obtained by glycerol and choline determination. These were of such a nature that they suggested the presence besides sphingomyelin of other lipids containing phosphorus that could not be determined after treatment acc. to SCHMIDT et al. As such lipids, if any, might have disappeared in the extensive purifications used by SCHMIDT et al and HACK the present author besides *purified* lecithins, cephalins and sphingomyelins studied also other lipid fractions. The results appear in table II. It may be seen that in the most alcohol soluble and the acetone soluble cephalin fractions and in the phosphatidyl colamine a considerable part of the phosphorus is not rendered acid soluble by 1 N KOH. Yet, the same fractions contain no or practically no choline. The P part in question cannot then be a constituent of *ordinary* sphingomyelin. The phosphatidyl ethanolamine had been dialyzed, so its contents of acid insoluble P was not due to some dialyzable contaminant. While it (the contaminant) could be a non-dialyzable one it seems more probable that it, owing to the thorough preparation and the solubility properties of the fraction (clear in chloroform and ether), is in lipid combination. On analysis the phosphatidyl ethanolamine preparation proved to contain about 50 % of the theoretical amount of ethanolamine. There are at present no reasons for the belief that any of the P of the ethanolamine cephalins is resistant to 1 N KOH followed by acid treatment. Thus, the KOH resistant part of the phosphorus in the preparation must be contained in some unknown lipid and, possibly, linked to sphingosine as in sphingomyelin i. e. a *sphingomyelin like compound having instead of choline another, reineckate*

non-precipitable, or no *N*-base. Liberation of choline from sphingomyelin during the preparation seems rather improbable owing to the known difficulty of its liberation by hydrolysis *in vitro*. Enzymatically this might take place in the tissues (cf. (chapter IX). In lipid fractionation coming in the cephalin fraction and being a choline free compound the supposed unknown lipid has been reckoned to the cephalin group and is in my tables denoted cephalins B (for its possible nature see also p. 21).

Also in fractionation experiments according to WEIL (table III and p. 77) the presence of a similar substance is suggested in fractions I and II, i. e. the most acetone or alcohol soluble, in both grey and, especially, white matter. It seems to be present in smaller amounts in other cephalin preparations in Table II besides those already described, diminishing in amount with decreasing alcohol solubility of the fraction. Some of it seems to be contained in the lecithin but none in pure phosphatidyl serine or the sphingomyelin preparations. SCHMIDT *et al.* (148) state that the cephalin preparations, where they found the phosphorus to be 100 % acid soluble after 1 N KOH treatment, had been twice recrystallized from hot alcohol in order to remove any sphingomyelin. Any choline free substance with KOH resistant phosphorus must also have been removed by this treatment.

*Standardizing* was carried out with each series and obeyed BEER'S law.

*Error.* By HACK put at  $\pm 1\%$ ; my own value was  $\pm 3.9\%$ .

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The method is an easily performed and rather exact one. According to the present knowledge it reveals the part of the P in phospholipids which is not bound to sphingosine, i. e. the part which is linked to glycerol (and to a small extent to inositol). At present it seems to be the only reliable method for sphingophospholipids (calculated by difference from total lipid P), HACK (79) having proved that reineckate precipitation — on which a formerly much used determination was based — is neither specific nor complete for sphingomyelin.<sup>1</sup>

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<sup>1</sup> While this book was in press a method convenient for the determination of sphingosine in lipids has been reported (124).

## Glycerol Determination.

Among the few micromethods for the determination of glycerol in lipids found by the author in the literature BLIX' (17) seems to be the most selective and least material requiring one hitherto developod. MACY (118) uses a periodate method which is selective for glycerol only if the extract is free from e. g. nitrogen bases and inositol. First and foremost, therefore, this method may be used to determine glycerol in phospholipid-free neutral fat. What was required in the present author's work was a method of estimating the total quantity of glycerophospholipids, the brain only containing negligible quantities of neutral fats. Actually BLIX' method is a way of determining alkoxyl groups in the extract. But if the lipid extract is pure, under the circumstances imposed by the method only lipid glycerol and to some extent hexose should react. The error arising from lipid hexose can be corrected by means of the cerebroside value (see Appendix on Methods) and the true total glycerol value will be obtained.

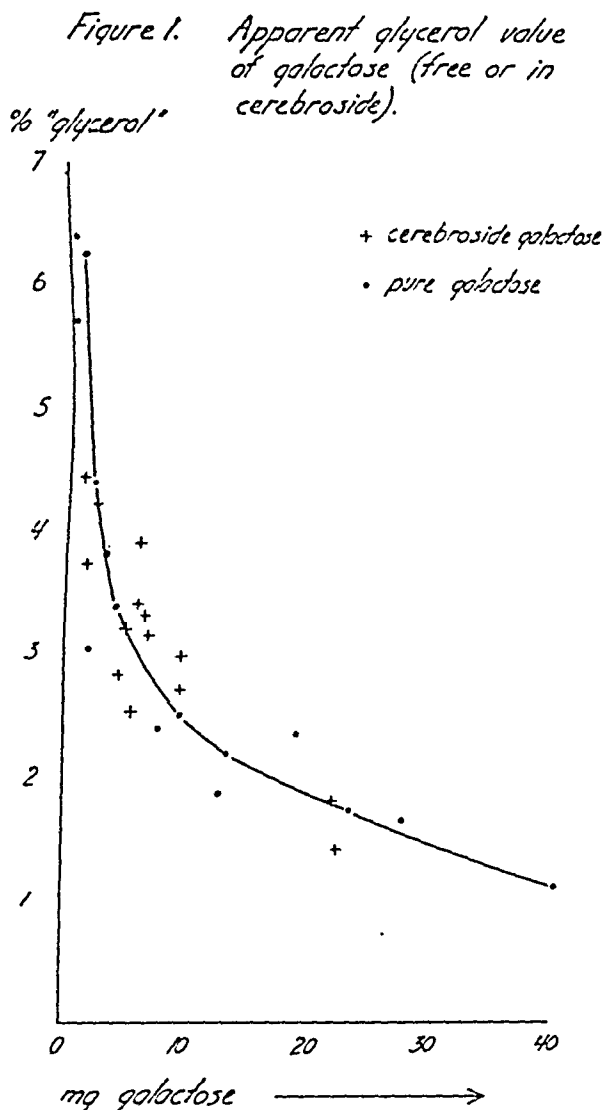
Certain details in the method deserve special mention. The *quality of the hydriodic acid* is of great importance. This has been emphasized by SCHMIDT et al. (149) 1945 who points out that each new package should be tested on samples containing known quantities of glycerol. PREGL's (140) instructions were followed in *storing the acid* and the *blank values* were not allowed to exceed 0.20 ml 0.02 N thiosulphate. Under the experimental conditions used by the present author his hydriodic acid gave a 100 % *recovery* of glycerol dissolved in acetic anhydride and of glycerol in purified cephalins and lecithin. BLIX had the same experience, FOLCH (67) had 95-96 % *recovery*, while the acid of SCHMIDT et al. must have been inferior since they obtained only a 75-85 % *recovery*. The method of *evaporating and drying the samples* was studied. Chloroform alone, evaporated in the usual way, gave no blank value. White matter chloroform extracts with or without an addition of 8 % ethanol were after evaporation dried, either in the usual way (see Appendix on Methods) or by repeated heating to 95° with repeated evacuations. The values obtained were in either case identical.

Accordingly the author's usual drying procedure completely removes ethanol (even in increased quantities), which otherwise would augment the glycerol values. The author routinely dissolved the samples in acetic anhydride before the determinations. This did not affect the results of the standardization nor did it increase the blank. Raising the *temperature* over the prescribed 120-125° to 130° did not significantly alter the analysis result.

In later years BLIX' glycerol method has become widely used in lipid analysis, especially for control analyses in lipid fractionation. So far, however, it has not been much tested in quantitative studies of lipids in biological materials. BLIX (18) studied the quantity of glycerol in plasmaphospholipids isolated by precipitation with acetone and  $MgCl_2$ . The present author determined the glycerol in unfractionated total extracts. In addition to the phospholipids such extracts contain other lipids. The influence of various lipids and lipid constituents on the glycerol value therefore was investigated.

SCHUWIRTH (156) observed that BLIX' method yields a value for cerebrosides. Their apparent glycerol contents was put at 0.84 %. He also found a »glycerol» value for glucose, amounting to 2.74 %. If the figure for cerebrosides is converted to be valid for their hexose component the value 3.82 % will be obtained. The present author tested galactose (SCHERING-KAHLBAUM) and found the apparent glycerol percentage to be dependent on the magnitude of the analyzed sample. On the basis of the results a curve could be constructed and it turned out that the apparent glycerol values for P free cerebrosides (prepared acc. KLENK (103)), converted to be valid for their galactose component, roughly followed the same curve. (see fig. 1 and 8 which was used for correction for cerebrosides in tissue extracts in glycerol determination).

Thus it is definitely established that *the galactose component and none other in the cerebrosides is responsible for the apparent glycerol value of cerebrosides*, probably owing to the formation of a distillable alkyl iodide. (Similarly glucose (30 mg) gave the value 0.77 %, mannose (40 mg) 0.54 %). No value was found for inositol, choline, serine, colamine and cholesterol. Fatty acids do not influence the determination.



fatty aldehydes were not tested by theoretically they should be inert.

Consequently *hexose* offers the only and well corrigible source of error for the method with respect to the lipid constituents that can occur in the chloroform reextracts. Naturally the presence in the extracts is possible of contaminants in the form of non-lipid glycerol or other reacting substances. Judging by the results of for example the dialysis and the formalin treatment it is, however, unlikely that they can be of much importance. The glycerol lipids

known in addition to neutral fat, which as mentioned before is negligible in nerve tissues, are phospholipids with respectively one and more glycerol molecules per atom of phosphorus. The presence of the latter has not been demonstrated in the nervous system but the occurrence of a phospholipid rich in glycerol was suggested by analysis results. In the fractionation experiment according to WEIL (table III and p. 77) its presence is suggested in especially the more acetone and alcohol soluble fractions. In concord with this finding the more alcohol and acetone soluble fractions of cephalin prepared according to FOLCH (65) in table II, contain much more glycerol relative to P than can be accounted for by monoglycerophospholipids. The molar proportion glycerol/KOH releasable phosphoric acid in phosphatidyl ethanolamine and in the acetone soluble cephalin was = 4:3 and 5:3, respectively. Thus the proportion is not very far away from that in cardiolipin according to PANGBORN (4:3) (135). It may be pointed out that the isolation of the cephalin fractions had as usual been preceded by thorough acetone extraction of brain and alcohol precipitation of the ether extract. Accordingly, any neutral fats present ought to have been largely eliminated. However, the possibility remains that the fraction in question contains partial decomposition products formed during the preparation of the phospholipids and that consequently much of the glycerol would be present as a diglyceride. Lastly, it is not altogether impossible that in addition to glycerol other alkoxyl groups, determinable by the glycerol method, could be bound in phospholipids, but at present nothing is known hereof.

*Standardizing.* See above.

*Error.* Calculated on 11 double determinations of different amounts of glycerol in acetic anhydride the error of a single determination was  $\pm 0.025$  ml. 0.02 N thiosulphate. The differences of a few double determinations on tissue extracts lay in the same range (1.43-1.43; 1.51-1.51; 3.54-3.50-3.48-3.53; 6.92-6.89). Thus, since samples using up at least 1 ml. thiosulphate were practically always taken, the maximal error was about  $\pm 2.5$  %.

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The method requires comparatively much material but at present the author knows no useful alternative. Applied as in the present investigation on nervous tissue lipid extracts it may be assumed to yield data on the amounts of glycerol lipids (if any minute traces of glycerophosphate present in the extract are disregarded). In addition to the known monoglycerophosphatides in the nervous system they could perhaps also include diglycerophospholipids and di- or triglycerides.

### Inositol Determination

In this field useful micromethods have been developed only during recent years. In 1943 PLATT and GLOCK (139) reported a wholly chemical method based on the consumption of periodic acid when that substance is used to oxidize inositol. Since, however, periodic acid reacts with several other substances (among the compounds occurring in lipid extracts, e. g. glycerol, hexose, serine, colamine), the determination in materials containing such substances must be preceded by an involved isolation procedure, and despite this the value obtained must be corrected for any contained glycerol. But with this refinement the results of the method are largely equivalent to those obtained by the microbiological methods of determining inositol. The latter are more convenient, require less material (a few  $\mu\text{g}$  (196) upwards of 200  $\mu\text{g}$  (12) while PLATT and GLOCK's method requires at least 500  $\mu\text{g}$  of inositol) and are in all probability more selective. Several different inositol requiring microorganisms have been utilized for the determination. WOOLLEY (196) as the first and later JURIST and FOY (90), WILLIAMS et al. (193) all used a strain of *Saccharomyces cerev. Hansen*; BURKHOLDER et al. (32), *Klockeria brevis*; BEADLE (12), *Neurospora crassa* inositolless. Unlike the last the two first organisms can grow, if only very slightly, on a medium free from inositol. They require a more composite medium than does the *Neurospora* which thrives in such simple an environment as one consisting of inorganic salts (containing  $\text{NH}_4$  ions), sugar and biotine. The growth of the yeast fungi is determined turbidimetrically while the rate of growth of *Neurospora* can be measured in special tubes and its total growth by weighing after drying, a more complicated method. The *Neurospora* requires greater quantities of inositol for exact determination,

as a rule a couple of hundred  $\mu\text{g}$ . Regarding *N. crassa* inositolless, BEADLE (13) in 1945 stated: »no other compound (than inositol) is known which will promote its growth.» Since then SCHOPFER et al. (155) have studied the organism and its reaction with *meso*-inositol and related compounds, isomers, derivatives, etc. Only *meso*-inositol significantly promoted the growth. Similarly, *Saccharomyces cerev.* has been shown to be about as selective as regards *meso*-inositol, provided that the environment contains optimum quantities of other growth promoting factors. However, it seems to some extent to be influenced by other substances contained in the samples than inositol.

Thus, *at the present time it would apparently be preferable to estimate inositol in lipid extracts by some microbiological method.* The proper organism to be used is a debatable question. The present author selected the more material requiring but probably most selective one, *Neurospora crassa inositolless*.

BEADLE's original method (12) was here altered on a few points. In studying the maximum growth curves for various doses of inositol it turned out that a maximum was obtained earlier (up to a few days) for higher concentrations (30-35  $\mu\text{g}/\text{flask}$ ) than for lower (10-15  $\mu\text{g}/\text{flask}$ ). For this reason the the proportionality as an ideal straight line between the inositol contents and the quantity of mycelium will not be obtained if the samples are incubated as briefly as prescribed by BEADLE (3 days), i. e. shorter than the period required for maximum growth at any concentration. If the maximum growths are estimated (see under Methods p. XX) a linear relation will on the other hand be obtained. Consequently the author has used the maximum growth method.

Since the author had in view to determine lipositol, which in itself has no growth promoting action on *N. Crassa inositolless* (12), the inositol in this compound must first be liberated. According to the findings of WOOLLEY (197) the maximum liberation of inositol from soy bean lipositol occurred after refluxing for at least 6 hours with 20 %  $\text{HCl}$ . The present author therefore used this *hydrolysis method*. On subsequent neutralization with a strong solution of  $\text{NaOH}$  the sample, whose volume is



kept at a minimum, will be almost saturated with NaCl. It turned out that the addition of NaCl in the quantities concerned to the medium for *N. c. inositolless* markedly affected its growth. For this reasons 1 ml of saturated NaCl solution was added to the standard series of inositol doses.

Among other substances phytin (Ciba) was used to test the *effectiveness of the hydrolysis*. By experience it is difficult to accomplish total liberation of inositol from this compound. Refluxing for 6 hours with 18 % HCl, BEADLE obtained only about  $\frac{1}{3}$  of the calculated quantity of inositol in determinable (=free) form. The present author, using WOOLLEY's hydrolysis, found 20.2 % inositol and 20.2 % P, both organically bound. This result is roughly equivalent to the theoretical amounts and in the proportion of 1 inositol molecule to 6 P atoms. The reason for the divergence from BEADLE's results is hard to understand. On lipid extracts from *corpus callosum* the same inositol result was obtained after 3 hours hydrolysis as after 6 or 12 hours. 3N HCl yielded somewhat lower results than 20 % HCl.

*Galactose, choline, aminoethanol, serine, glycerol, sodium glycerophosphate* in the maximum quantities with which they usually occurred in the author's extract samples *did not* themselves *promote the growth of N. c. inositolless*, nor did their presence significantly influence inositol induced growth.

Lastly, it was attempted to further check the specificity by studying the gammexane inhibition on the growth of *N. c. inositolless* on lipid hydrolysates, gammexane being a compound considered a possible structurally analogous competitive inhibitor of *meso*-inositol (27): equivalent doses of inositol and lipid hydrolysates were in a like measure inhibited by gammexane.

*Standardizing.* By the author's maximum growth method including NaCl addition a linear correlation was obtained between the dosis of inositol and the amount of mycelium for inositol quantities between 15 and 35  $\mu\text{g}$ .

*Error.* BEADLE maintains that his development of the method is accurate within about 0.3 mg of mycelium at an inositol concentration of 20  $\mu\text{g}/\text{flask}$ . The present author's experience of BEADLE's method was not as satisfactory. With the modifica-

tions mentioned in the foregoing the error of method was certainly considerably lower than  $\pm 5\%$ .

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The inositol method adopted cannot be objected to from the point of view of selectivity. The relatively large material requirements are a disadvantage in respect of certain tissues studied by the author (grey matter), and in such cases some of the more sensitive and quicker methods should be tried.

### Choline Determination

Being a long since known chemical compound of considerable biological import, choline has been subjected to numerous attempts at exact determination. In so doing its occurrence in nature in a free state has been far more difficult to evaluate quantitatively than its occurrence in phospholipids. This is partly due to the fact that the amount of free choline in tissues is much lower than the bound quantity, and partly because biologically occurring substances may disturb the hitherto tested methods of determining choline and in the main being water soluble they especially affect the determination of free choline. While thus determination of lipid choline should be easier to perform, adequate methods have nevertheless been difficult to develop. Owing to the great importance in the present author's investigations of the lipid choline, a fairly full discussion of the problems involved in its determination must be considered necessary.

The first step in the determination of lipid choline is always its liberation. There have been adopted for the hydrolysis various acids (168) or bases, in aqueous or alcoholic environment (118), and heating at atmospheric or higher pressures (87). The liberated choline has then been determined by chemical methods or biological assay procedures. Of the latter pharmacological or microbiological methods, especially, are useful. *Pharmacologically*, choline is determined after acetylation by quantitative measurements of its effect on e. g. rabbit intestine. BORGLIN's modification (22) of this method seems to belong to the more sensitive and specific ones but its standard deviation is apparently high even in the hands of investigators well cognizant of pharmacological technique.

More reproducible results despite very small sample quantities may be had *microbiologically* by assay with the *cholineless* mutant of the mould *Neurospora crassa* (87) but by this method the specificity becomes relatively low since in addition to choline and ammonium bases chemically related to choline the growth is promoted also by methionine and unhydrolyzed lecithin, i. e. in general compounds supplying »labile methyl». Neither are the *chemical methods* available characterized by absolute specificity for choline. Two principles have chiefly been used for microdeterminations. The first, involving primary precipitation of the choline as a complex iodine compound, was reported by ROMAN (146) and published in a recent modification by ERICKSON et al. (55); HANDLER 1947 (85) considers it »not entirely satisfactory» and inferior to the one founded on the second principle. This implies the primary precipitation of choline as reineckate followed by some sort of colorimetric estimation. Various modifications of the reineckate method differ essentially as to the precipitation conditions, the means of purifying the precipitate and the preparation for colorimetry of the pure choline reineckate.

As was mentioned in passing there are *other bases than choline*, inorganic as well as organic, which *may be precipitated with reineckate*. *Metals* that can be precipitated with  $H_2S$  in acid solution (e. g. Cd) form slightly soluble reineckates (39).  $BaCl_2$  tested in saturated solution gives a reineckate which is easily soluble in water. Consequently it implies no source of error when  $Ba(OH)_2$  hydrolysis with HCl neutralisation is used. Numerous *organic N-bases* form reineckates. Only those compounds, however, which contain tertiary or quaternary alkylated N-atoms form reineckates about as difficultly soluble in water as choline reineckate. Thus, the solubilities (cf. GUGGENHEIM (75)) in water at room temperature for the reineckates of some substances are given as follows: methyl guanidine 0.43 %, histidine 0.3, betaine 0.28, creatine 0.16, creatinine 0.16, carnosine 0.16, carnitine 0.10, choline 0.02, acetylcholine 0.018, tetramine 0.003; in 10 % HCl the solubility of the reineckates of betaine, carnitine and choline are respectively 0.2, 0.15 and 0.03 (172). Numerous substances of this type occur in the animal world. DU VIGNEAUD (46) lists

some 30 naturally occurring N-methyl bases, among them 13 quaternary, and even more exist. Preferably, they occur in water soluble form, and as a rule in insignificant quantities in relation to the quantities of phospholipid choline in the same tissue. But probably many of them have such solubility properties that they can pass over into a lipid extract. Together they may then possibly become a significant source of error in the determination of the choline. In fact, this seems proved regarding some tissues by means of experiments carried out by ENTENMAN et al. (53) and GLICK (73). Lastly, it is not impossible that N-methylated and reineckate precipitable substances other than choline are constituents in some phospholipids (38, 24).

If choline exclusively is to be determined the sources of error mentioned in the above must therefore as far as possible be eliminated. Except by refined extraction of the lipids, excluding water soluble contaminants (see above, p. 37), this goal has been striven for by utilizing *selective precipitation and purification of choline reineckates*. GLICK (73) points to the above illustrated fact that the solubility of betaine reineckates is greater in comparison to that of choline reineckates at high pH values. He therefore advocates a pH between 8 and 10 for precipitation of choline samples. Choline reineckate is deposited practically quantitatively in environments with a pH lower than 10 and excess of Reinecke salt where the solubility of the choline reineckate will be 0.0015 %. As a selective, choline preserving rinsing agent for the reineckate precipitate WINZLER and MESERVE (195) used a saturated aqueous solution of choline reineckate. Using such principles one should be able to fairly exactly and selectively determine choline in lipid hydrolysates from nervous tissue.

When the author faced the choice of choline method for the present studies it seemed most profitable to employ a reineckate modification which he himself had used earlier in studying plasmalipids (26) and later had been adapted for 0.1-0.6 mg choline. It involved hydrolysis with 0.1 N NaOH in ethanol, precipitation of liberated choline with slight excess of Reinecke salt at pH 2-3, rinsing with iced water and alcohol, and colorimetry of the reineckate in acetone solution. The adopted

hydrolysis method had been demonstrated more effectively than NaOH in water or HCl in methanol to separate choline from lipids. The difference seemed to be due to relative ineffectiveness of the latter two hydrolytic agents on sphingomyelin (tested on protagon from brain). Later, reports have appeared (ENTENMAN et al. (54)) indicating that  $\text{Ba}(\text{OH})_2$  in saturated aqueous solution will still more completely liberate choline from lipids. Hence, the author decided to reinvestigate the action on brain choline lipids of some hydrolytic agents.

Firstly, the method mentioned above of ENTENMAN et al. (54), besides  $\text{Ba}(\text{OH})_2$  hydrolysis also including precipitation with a great excess of Reinecke salt at 1.2 N HCl concentration, rinsing with 1.2 N HCl and colorimetry in acetone solution of the reineckate, was rendered useful for work on a microscale (see Choline method, p. XIII). Then it was used in parallel with the author's *alcoholic NaOH* method on extracts from the nervous system. The results appear in table 1.

*The ENTENMAN method evidently yields clearly higher values than determinations according to BRANTE.* The absolute difference is greatest in material rich in myelin while in most cases it is insignificant in grey matter. This circumstance *parallels the sphingomyelin contents* of the respective tissues and hence the difference may be suspected to be somehow allied with this choline fraction. Such being the case it was *a priori* most probable that BRANTE's hydrolysis method did not completely set free the choline in sphingomyelin. This was tested on brain sphingomyelin purified without alkali treatment. The result of the double determination expressed in mol. equivalents in comparison to sphingomyelin P, which amounted to 2.97 % of the substance, was according to ENTENMAN = 0.97 and according to BRANTE = 0.81. Consequently the latter method was about 84 % as efficacious as the former, and it therefore appeared that the above mentioned difference was at least partly, but scarcely altogether, explained. In the below table choline according to BRANTE is in white matter but 71 % of the value according to ENTENMAN but if the lecithin choline included in both values, which is quantitatively liberated not only by  $\text{Ba}(\text{OH})_2$  but also by mild KOH treatment (cf. p. 59) and in all probability also by alcoholic NaOH according to BRANTE,

Table 1: *Choline in Nervous Tissue Extracts Determined According to Entenman et al. and Brante, respectively*

Material	Choline in mg/ml			
	Acc. to Entenman et al.		Acc. to Brante	
I. Alcohol-ether extract from whole brain (mean of 12 determinations)	0.314 $\pm$ 0.013		0.280 $\pm$ 0.011	
Material	Choline %			
	Grey matter		White matter	
	Acc. to Entenman	Acc. to Brante	Acc. to Entenman	Acc. to Brante
II. Various chloroform reextracts from nervous tissue				
Man 1 (brain)	0.26*	0.25*	0.51	0.31
" 2 "	0.21	0.13	0.45	0.34
" 3 "	—	—	0.53	0.34
Cow 1 "	0.23	0.20	0.43	0.34
" 2 "	0.25	0.17	0.42	0.35
" 3 "	0.21	0.20	0.35	0.26
" 4 "	—	—	0.78	0.54
" 5 (spinal marrow)	—	—	0.73	0.52

\* mean of 5 determinations

is subtracted from the values in the above table it turns out that the sphingomyelin choline in white matter according to BRANTE is less than 45 % of the value according to ENTENMAN (computed for white matter mtrls. nr. 3 and 5 where KOH choline was determined).

But the two tested methods did not differ only in the hydrolysis but also in the precipitation and purification conditions. The strongly acid environment and the large excess of  $\text{NH}_4$ -reineckate in ENTENMAN's method probably both act in favour of codetermination of other reineckates besides that of choline. The first mentioned factor and the presence of Ba ions can be assumed to bring about the separation of the fatty acids in such a way as to allow the choline to be more completely re-

leased to the water phase. Lastly, the presence of  $\text{BaCl}_2$  could somehow increase the precipitation of choline or any other reineckate. The latter alternative as regards choline could, however, immediately be disregarded since standard curves with or without  $\text{BaCl}_2$  were identical. Several methods were applied to attempt the solution of the other problems.

Thus, one experiment was carried out using ENTENMAN'S determination method on BRANTE'S hydrolysate and vice versa. In so doing alcoholic NaOH hydrolysate was acidified to 1.2 N HCl concentration, 150 mg  $\text{BaCl}_2$  was added and for the rest the sample was treated according to ENTENMAN;  $\text{Ba}(\text{OH})_2$  hydrolysate was neutralized with  $\text{H}_2\text{SO}_4$  to pH 2-3, Ba ions thereby largely being removed, and thereafter treated according to BRANTE. Results of parallel determinations on extracts from white matter (computed by means of standard curves, made up simultaneously and separate for each method) appear in Table 2.

Table 2

White matter from:	$\text{Ba}(\text{OH})_2$ hydrolysis according to Entenman		Alcoholic NaOH hydro- lysis according to Brante	
	Determination as usual acc. to Entenman	Determination acc. to Brante	Determination as usual acc. to Brante	Determination acc. to Enten- man
Cow I	0.35	0.34	0.26	0.31
" II	0.43	—	0.34	0.46
" III	0.43	—	—	0.32
" IV spinal marrow	0.73	0.64	0.52	0.80
" V	0.78	0.62	0.54	0.63
Man I	0.57	0.45*	0.31	0.44
" II	0.51	—	0.31	0.40

\* Rinsing performed with saturated choline reineckate solution

*Evidently the alcoholic NaOH hydrolysate values are consistently much higher when determined under ENTENMAN'S conditions than according to BRANTE. In some cases they come up to or even exceed the values in  $\text{Ba}(\text{OH})_2$  hydrolysates determined according to ENTENMAN. These latter are on the other hand somewhat higher than the values for the same hydro-*

lysates determined according to BRANTE. Taken together the results demonstrate that *after completed alkaline hydrolysis the continued treatment is of great importance for the results of reineckate determinable substance.*

The reason for the higher values obtained with ENTENMAN'S treatment after the hydrolysis was considered. Firstly, the HCl concentration might have an additional hydrolysing effect or accomplish a more effective extraction of liberated choline. However, it appeared that the HCl concentration alone could not be very important as filtering at pH 2-3 did not influence the result of determinations according to ENTENMAN, and determinations according to GLICK after neutralization to pH 8-9 with rinsing according to BRANTE did not yield significantly lower values. (GLICK (73) had similar results.) The presence of Ba ions and the excess of reineckate does not as mentioned affect the pure choline value. Therefore the conclusion is arrived at that *either some factor prevents complete determination of the choline present, or otherwise there must be some other reineckate precipitable substance in addition to choline.* In the former case Ba ions and Reinecke salt in excess would reduce or eliminate the factor in question, in the latter they would be essential for the determinability of the substance. These matters require further investigations.

At this point the present author set out to investigate if any of the bases known in lipids or any N-methyl compounds that can occur in connection with lipids could have caused the described discrepancies. Sphingosine was *a priori* considered inconceivable in this connection (cf. also the experiment with purified sphingomyelin, p. 54). Colamine, mono- and dimethylaminoethanol, serine, sarcosine, trimethylamine, creatinine, adrenaline and thiamine, all in quantities of 0.5-1 mg gave no values after  $\text{Ba}(\text{OH})_2$  treatment in determinations according to ENTENMAN. Betaine yielded equivalent extinction values by ENTENMAN'S and BRANTE'S methods, viz. on a molecular basis about 30 % of that of choline. Similarly, acetylcholine gave about 90 % of the choline extinction for both methods, while the corresponding figures for neurine and trimethylamine-oxide in repeated experiments was about 124 % and 30 %, respectively, according to ENTENMAN and 106 % and 0, respectively, according to BRANTE. *So, neurine and trimethylamineoxide were substances giving greater values, calculated as choline, under ENTENMAN'S conditions than under those of BRANTE, but the difference is small.*

While the experiments hitherto described do not prove the presence in the lipid extracts of any other substance precipitable by reineckate than choline it seems probable that *the values obtained by ENTENMAN'S method of determining choline include all, or practically all, the choline that can be released from lecithin and sphingomyelin in brain.* In fact, the present



author found 97 % and probably 100 % of the theoretically present choline per P atom in pure sphingomyelin and lecithin, respectively, to be determinable by the method described in the present work (cf. Methods, p. XIII). The sphingomyelin figure should be contrasted with HACK's (79) who, using similar methods of determination, even after 24 hours hydrolysis of sphingomyelin preparations found considerably lower choline contents than the theoretical. The reason for this disparity is not clear. In addition to the ones mentioned the author has tried some *other hydrolysis methods*, viz. refluxion with 3 N HCl, yielding values similar to those with alcoholic NaOH, and  $\text{Ba}(\text{OH})_2$  in alcohol according to ERICKSON (118), which gave varying results at best comparable to those with  $\text{Ba}(\text{OH})_2$  in saturated aqueous solution according to ENTENMAN. Hence, the last mentioned method is the one that has given the highest and most consistent figures. Carefully keeping in mind the possibility of codetermination of other substances than choline, the present author adopted it in his own investigations.

The method according to ENTENMAN et al. was modified but in as few respects as possible; thus it was developed to exactly determine much smaller samples, viz. 0.1-0.6 mg choline, by using smaller volumes in the different steps and colorimentering in longer cuvettes. A marked tendency to cloudiness in the final acetone solution of the choline reineckate could be eliminated by thorough centrifuging and careful decanting. This cloudiness was more difficult to remove completely if rinsing and acetone dissolving was carried out on a glass filter — the method first attempted. Rinsing on glass filter with saturated choline reineckate solution according to WINZLER and MESERVE (195) did not give significantly different results as compared to rinsing with 1.2 N HCl. (If desired this may be interpreted as pointing *away from* the presence in the precipitate of reineckates other than that of choline in significant amounts.)

As previously mentioned the colorimetry was performed in microcuvettes. The absorption was measured at 540 m $\mu$ . Greater extinctions could have been obtained at 327 m $\mu$  or by using MARENZI and CARDINI's (195, 120) method for determining chromium in reineckates. However, the method adopted was satisfactory and the difficulties encountered in adapting the

method for such small choline quantities as the ones used lie, as pointed out by HANDLER (85), *before* the colorimetical procedure. If these difficulties could be eliminated (see below) the modifications mentioned would probably become more useful for the determination of reineckates.

*Standardizing and recovery experiments.* As a rule the standardizing gave a perfectly linear curve for choline amounts ranging from 0.15 to 0.60 mg. 98-100 % recovery was obtained in determinations on standard samples that had also been subjected to the hydrolysis procedures, both the one with  $\text{Ba}(\text{OH})_2$  and the one with alcoholic  $\text{NaOH}$ . Additions of pure choline (0.3 mg) to lipid samples were also quantitatively recovered. The heating (see p. V) of the lipid samples during evaporation and drying proved to be without importance for the choline result.

*Error.* Calculated on 17 double determinations of choline in different nervous tissue extracts the error of a single determination was  $\pm 0.013$  mg., i.e. for the usual amount of about 0.300 mg choline per sample  $\pm 4.3$  %. For pure choline solutions the error was of the same order.

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The method is rather time consuming but also sensitive and reproducible. In addition to all lipid choline the values obtained probably include most free choline (see p. 35) and perhaps also other reineckate precipitable compounds in lipid linkage or not, but certainly in small quantities. Hence, *the method may be considered to give maximum choline figures.* A possible means for the more selective determination of choline in yet smaller quantities might in the view of the present author be to separate the hydrolysate on a paper chromatogram, indicate by iodine (24), excise the spot and determine by microbiological means or following reineckate precipitation according to WINZLER and MESERVE.

### Determination of Lecithin Choline

In 1947 HACK (78) demonstrated that the same treatment with 1 N  $\text{KOH}$ , which according to SCHMIDT et al. splits off selectively to acid soluble form phosphorus from non-sphingolipids, also involves a selective release of choline from the same lipids. Judging by SCHMIDT et al.'s (148) investigations

it is evident that 1 N KOH splits glycerophospholipids into free fatty acids, glycerophosphoric acid and free bases. Its action on sphingomyelin, however, in some respects involves problems. Surely choline phosphoric acid ester is not liberated, for this would imply acid solubility of the sphingomyelin phosphorus after such treatment, which is not the case. It is doubtful whether so mild alkali treatment can liberate amide linked fatty acids (143). Clearly, the choline is not liberated in free form. This is surprising, for it is not known that choline is differently linked in sphingomyelin than in glycerophospholipids, i. e. esterified to phosphoric acid. HACK gives no explanation; in passing he simply mentions that sphingomyelin unlike other lipid fractions was insoluble in the hydrolysis agent. The present author considers it improbable that inaccessibility to the hydrolysis agent can explain the non-releasability of sphingomyelin choline. Even a fine suspension of sphingomyelin yields no choline. Under such circumstances at least a portion of it should be accessible to the hydrolysis agent. If the explanation discussed can be disregarded, the *choline must be assumed somehow protected against the KOH effect owing to the specific structure of sphingomyelin.*

While the mechanism underlying the determination, consequently, is an open question, HACK's data based on analysis of among other substances  $\text{CdCl}_2$ -lecithin from eggs, lecithin from heart and sphingomyelin from brain were found correct for purified lecithin and sphingomyelin obtained from cow brain (see table II for results).

As regards method it may be mentioned that the present author largely has followed the principles laid down by HACK. The HCl concentration before the filtration was, however, put at 1.2 N instead of about 0.4 N and after the filtration the determination was carried out strictly as described for the determination of total choline. As may be seen in the foregoing these alternations in no way affected the results on pure lecithin or sphingomyelin. Just as little as when glycerol phosphorus was determined did raising the temperature to  $45^\circ$  or lowering it to  $34^\circ$  during the hydrolysis affect the choline value, nor did prolongation of the hydrolysis to 48 hours. *Routine hydrolysis would thus appear to be complete. Addi-*

tion before filtration of 150 mg  $\text{BaCl}_2$  per sample to lipid hydrolysates from white matter gave a slight increase of the value (about 8 and 10 %, respectively, in double determinations in two instances). This may suggest the presence in these extracts of some factor inhibiting the precipitation of choline or else some substance capable of being determined as reineckate only in the presence of Ba (cf. discussion in total choline determination p. 57). Thus, it is conceivable that the values for lecithin in some tissues are too low, for  $\text{BaCl}_2$  was not added in the routine determinations.

*Standardizing.* Cf. total choline method.

*Error.* This seems to be of the same order as that for total choline determination. 7 double determinations on different lipid extracts had the error  $\pm 0.008$  mg., i. e.  $\pm 4$  % of the in analysis generally used amount, 0.200 mg choline.

Everything indicates that the method adopted is selective and seemingly quantitative for lecithin when determined in nervous tissues. Some additional points of interest in this connexion are discussed under the total choline method.

### Determination of $\text{NH}_2\text{-N}$ Bases

In many earlier publications  $\text{NH}_2\text{-N}$  determination in a lipid extract hydrolysate has been used as a measure of its contents of cephalins, provided that the only base contained in them was ethanolamine. But later it turned out that on the one hand it was difficult to remove impurities containing  $\text{NH}_2\text{-N}$  from a lipid extract, and on the other that besides ethanolamine considerable quantities of serine (65) is linked in the cephalins. This imposed the necessity of more specific methods, and micromethods for ethanolamine and serine in lipid analysis have already been adopted by several investigators. All these methods include hydrolysis, the performance of which deserves special mention.

The present author required a *hydrolysis method* of simultaneous use for ethanolamine, serine and inositol analyses. For the latter (see inositol method, p. 49) refluxing with

6 N HCl for at least 6 hours was essential. Judging by data in THIERFELDER and KLENK's monography on lipids (182) such a procedure would quantitatively release all base from cephalins, and it was therefore adopted in the present investigation. ARTOM (9) has found 6 N HCl methanol to be a satisfactory hydrolysis agent. In the hands of the present author it was found a little less effective than 6 N aqueous HCl for pure phosphatidyl ethanolamine and phosphatidyl serine. BURMASTER (33) found 20 % HCl in 50 % alcohol satisfactory. EDMAN and ÅQUIST (49) used 2 N aqueous CHl and maintain that it is quantitative for ethanolamine liberation. The present author has not systematically studied the adequateness of the hydrolysis procedure used, but judging by the above statements it should be satisfactory. Alkaline hydrolysis has repeatedly been found to result in losses (e. g. ARTOM (9)).

**Ethanolamine.** In 1945 ARTOM (9) utilized the formation of  $\text{NH}_3$  in periodate treatment of ethanolamine and serine for their quantitative determination in lipids. The  $\text{NH}_3$  was estimated in a hydrolysate before and after a permutit adsorption procedure. The latter quantitatively removed the ethanolamine whose  $\text{NH}_3$  was calculated by difference. However, the amounts required for determination were relatively large. Some disturbing — value augmenting — factor seemed to be present in materials containing much sphingolipids. The method is specific for compounds having a hydroxyl group and an amino group on adjacent carbon atoms. A microdiffusion modification has been used by BURMASTER (34).

In 1941 BLIX reported a probably even more specific principle for the determination of ethanolamine (18). The ethanolamine in a hydrolysate is distilled off selectively into dilute HCl and its amount estimated by back titration with dilute NaOH. The sensitivity and specificity of the method were improved by EDMAN and ÅQUIST by introducing a colorimetric method (Berthelot reaction), specific for  $\text{NH}_3$  and primary amines, for determination of the ethanolamine in the distillate. The present author adopted the distillation apparatus in the work last mentioned, but used  $\text{NH}_2\text{-N}$  determination according to VAN SLYKE (137) instead of the colorimetric procedure. The

VAN SLYKE method is nearly as sensitive as the latter and serves to further exclude any disturbing action of  $\text{NH}_3$  not driven off by predistillation at a relatively low temperature.

Since strong  $\text{HCl}$  was used in the hydrolysis, and in the neutralization with saturated  $\text{NaOH}$  solution to keep the volume as low as possible, the resultant solution for estimation was almost saturated with  $\text{NaCl}$ . *Tests with ethanolamine, alone or mixed with amino acid or  $\text{NH}_3$  in saturated  $\text{NaCl}$  solution, showed the ethanolamine to be quantitatively recovered.*

Determinations on purified phosphatidyl ethanolamine yielded values near to the theoretical ones.

For alkalization before distillation either  $\text{NaOH}$  according to EDMAN and ÅQUIST or a  $\text{CaO}$  dispersion according to BLIX could be used with equal success. Instead of EDMAN's heating bath with WOOD's metal an ordinary large water bath was found more practical.

Error was not determined but seemed to be a few % for pure ethanolamine as well as lipid samples (cf. EDMAN and ÅQUIST).

The ethanolamine determination employed is satisfactorily sensitive, and seems highly specific, the only possible source of error being unsubstituted amines with similar distillability as ethanolamine. With  $\text{NH}_2\text{-N}$  determination in the distillate it should not codetermine any mono- or dimethylaminoethanol, but, since these compounds distill at higher pressures than ethanolamine, they must if they are present in the hydrolysate be contained in the resulting distillate. Any non-correlation between the  $\text{NH}_2\text{-N}$  value and the base titre value in the distillate may make their existence suspected and might be developed into a method for their determination. The ethanolamine value represents the quantity of ethanolamine phospholipids, including phosphatidyl ethanolamine and any others (e. g. plasmalogens).

Serine. ARTOM's procedure has already been described in the above. It is unduly insensitive but specific for certain hydroxyamino acids. Less specific but more sensitive is the method proposed by VAN SLYKE et al. (161) employed in the present investigations in which are estimated  $\alpha$ -aminocarboxylic acids. When applied to lipid samples free from contaminants it specifically gives their lipid bound amino acids, i. e. according to our present knowledge serine. The method has

been adopted by several authors for determining amino acids in fractions in the preparation of various lipids, but the author has not yet seen it used in quantitative studies of amino acids in tissues. And, in fact, the method is inconvenient unless a lipid extract is used which is perfectly free from impurities containing amino acids. Therefore, the few results as to the serine contents in nervous tissues given by the present author must be regarded as certainly being too high.

The VAN SLYKE ninhydrin method has been used *unmodified*. Since the samples for determination were contained in almost saturated NaCl solution *recovery experiments* were made with pure alanine and serine in such solution. The recovery was quantitative.

*Error.* According to VAN SLYKE et al. (83) it amounts to  $\pm 1\%$  at 0.04 mg quantities of amino carboxyl N per sample. The present author had similar experiences.

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The method is complicated and time consuming, but sensitive, exact and selective for amino acids. It requires a high degree of purity of the lipid extract, a purity certainly not attained by chloroform reextraction as in the present study. This source of error might be excluded by a separation by means of paper chromatography before determination.

In the present study *a better value of the serine phospholipid contents* is perhaps obtainable by the *difference*: (total phospholipids—[lecithin + sphingophospholipids]) — ethanolamine phospholipids. Besides phosphatidyl serine the result may include unknown amino acid phospholipids.

### Cholesterol Determination

To the present author the method developed by SCHOENHEIMER-SPERRY seemed the most tested and accurate one in this field. As an advantage, it also affords a simple means of determining any esterified cholesterol. Doktor SPERRY had the kindness to supply me with the latest description of his method (dated 1945), and I have followed it fairly closely; the use of a ready-mixed, chilled acetic anhydride-sulphuric acid reagent was tried and found satisfactory, provided the experimental series could be read off within 1 hour after mixing. For the large experimental series of the present author this was practical

only if an electrophotometer was employed for the colorimetric determinations. The readings of an electrophotometer are, of course, seriously affected by cloudiness in the solutions. As it proved difficult always to avoid some single flocculation, the author went in for the old, more time consuming method with separate addition of anhydride and sulphuric acid and reading in a Pulfrich photometer where abnormal changes in the solution are immediately detected.

After carefully testing a number of different cholesterol, acetic anhydride and digitonin preparations the author finally found satisfactory reagents (see p. XXIII). However, in each test series it proved necessary to include a blank thereby compensating for the minute but quite perceptible extinction caused by the digitonin preparation used.

For the determination the present author used aliquots of his chloroform extract. Unlike the acetone-alcohol extract generally used by SCHOENHEIMER-SPERRY it holds a great proportion of other lipids than cholesterol. This might have a disturbing influence but such was rendered improbable by fact that *the cholesterol value in the acetone-alcohol reextract of the chloroform reextract did not significantly differ from the direct value in the latter*. In the lipid fractions obtained according to WEIL from nervous tissue (p. 76) practically all the cholesterol could be recovered in the first acetone extract. The combined value of all the other fractions was less than 1 % of the value in the acetone fraction. In the alcohol fraction a low value was obtained for the unhydrolyzed sample and nil for the hydrolyzed one. This proved that *in addition to cholesterol other colourific substances can occur but their quantity is minute* which, besides, indirectly appears from the fact that the free and total cholesterol values were identical for practically all normal nervous tissues.

*Standardizing.* With the reagents finally adopted a standard curve was obtained which exactly obeyed LAMBERT-BEER's law. Its slope altered but insignificantly from series to series for long periods.

*Error.* As only a few double samples were determined, the standard error of a single determination had to be calculated



on the differences between the value for free and total cholesterol in white matter, where the bound cholesterol was = 0. Based on 30 such differences the error was  $\pm 0.0028$  for a mean value of 0.0756 mg cholesterol, i. e.  $\pm 3.7\%$ .

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Thus, the method was found highly sensitive and well reproducible. It also seems to be highly specific.

Using some precautionary measures the method including ready-mixed reagents could perhaps be electrophotometrically applied. This would imply a saving in time.

### Cerebroside Determination

For micro-quantitative *determination of cerebrosides* mostly the *reductive power of their hexose part* has been utilized. The reduction value has been determined by a modified FEHLING-method (184), with ferricyanide followed by subsequent thiosulphate titration of residual unreduced ferricyanide according to HAGEDORN-JENSEN (KIMMELSTIEL (94), FAWAZ et al. (57)) and, most recently, by oxidimetric titration with cerium sulphate of the reduced ferricyanide according to MILLER and VAN SLYKE (BRAND & SPERRY (23)). Since in the latter modification the hexose value is obtained directly in only one exact titration and the proportionality between sugar amount and consumption of titre fluid is accurately constant over a wide range, the method constitutes an improvement of the HAGEDORN-JENSEN procedure. It in addition involves a well developed hydrolyzing technique which is quantitative for hexose in cerebrosides. Most previously used hydrolysis methods have evidently been incomplete or partially destroyed the hexose. By the introduction of a determination of the reduction in unhydrolyzed extract sample, the value obtained for hydrolyzed sample is corrected for the presence of any contaminating, reducing non-cerebroside components. KIMMELSTIEL (94) among others has pointed out the necessity of taking this precaution.

During recent years methods have been developed also to make possible *colorimetric determination of the lipid hexose*.

BRÜCKNER (30) determined lipid galactose by means of an orcine reaction especially developed for the purpose; EDMAN (48) did so using a karbazol reaction. Reportedly it is possible with the aid of the extinction value in two different wavelength bands to form some opinion not only of the galactose level but also of the concentration of other hexoses. OTTENSTEIN, SCHMIDT and THANNHAUSER (unpublished, see (181)) have also used a quantitative method of separately determining glucose and galactose in cerebroside.

*Thus, when they are as best, the titrimetrical methods of reduction may be used to determine the total quantity of releasable hexoses in lipids while the colorimetrical methods in addition yield a differentiation between glucose and galactose in cerebroside.* In certain cases the latter methods therefore may be of great value (e. g. in studying cases of MORBUS GAUCHER) but it has been found that *the hexose in brain cerebroside is practically entirely galactose.* (KLENK (100), confirmed by the present author (24)). This is true also in cases of MORBUS GAUCHER, where the cerebroside deposited in the reticulo-endothelium contain glucose. Such being the case *the titrimetric and colorimetric procedures ought to be equivalent in analyzing nervous tissues.* Probably other sugars in addition to galactose occur in small amounts in the gangliosides but in comparison to the cerebroside-hexose their quantity must be so minute, even in grey matter, that they are of little importance for the hexose lipid value obtained by the reduction method. On the other hand the orcine reaction may possibly be deleteriously influenced by the neuraminic acid in the gangliosides, thereby giving rise to errors in the determination of galactose and glucose lipids. Since the orcine method appeared less conclusively tested when the present author commenced his experiments he chose to use the method developed by BRAND & SPERRY.

Few deviations only have been made from the original instructions for this method. Instead of stoppered flasks the author used 10 ml ampoules that could be sealed by melting. A lower blank was obtained if the hydrochloric acid for the hydrolysis was redistilled. The filter papers were simply boiled instead of Soxhlet treated with distilled water. By these means

the blank value for acid hydrolyzed samples could be kept down to 0.25-0.40 ml cerium sulphate, for unhydrolyzed ones to 0.08-0.13 ml. MILLER and VAN SLYKE'S original indicator, Setopaline C, was used. Emulsification with alkyl sulphate was not used. Like BRAND & SPERRY the author in carrying out determinations on tissue extracts sometimes obtained rather much cloudiness in the unhydrolyzed samples. This cloudiness is often difficult to remove by the aid of the recommended refiltrations and it may to some extent make it hard to see when the titration is concluded. MACY (118) recommends the addition of NaCl before the clarification in order to reduce the final opalescence. The author has, however, not tested this possibility.

By means of determination on known galactose and glucose solutions of various strengths with or without boiling with acid or water it was demonstrated that none of these hexoses are destroyed during the hydrolysis provided that perfectly clean vessels are used.

The author got some suspicion that during the clarification fatty acids to some extent passed through the filter as soaps and then had a disturbing — reducing — action. For in determining reduced substance in very large quantities of purified and dialyzed phospholipids (25 mg colamine cephalin per sample) unreasonably high values were obtained whether or not the sample was hydrolyzed. It could be proved that ether extraction of the samples before the clarification eliminated most of this reduction. In practice a cerebroside sample from nervous tissues contained at most 4-5 mg totally of phospholipids. In order to eliminate the possibility of a disturbing influence hereof, the reduction in samples, with the fatty acids removed by ether extraction before the clarification, was compared to that in samples, with the fatty acids left as usual. The experiment was carried out on both white and grey matter and on fetal brain in which latter some authors (125, 104) in contradistinction to the present one had been unable to find any cerebroside. After the ether extraction the samples were absolutely clear. However, clarification was carried out as usual. After evaporation and dispersion in water the ether extracts also gave reduction values. Results:

Extract	Value of ordinary direct de- termina- tion % fresh tissue	Value aft- er ether extraction of fatty acids % fresh tissue
White matter spinal cord, cow	3.20	3.02
Brain cortex, boy 10 years old	1.25	1.22
Brain cortex, fetus 8 months old	0.20	0.21

Consequently, *the presence of fatty acids and unhydrolyzed lipids during the clarification under ordinary circumstances have little or no influence on the reduction value.* Nor did cerebroside fatty acids have any effect in experiments on purified cerebrosides.

Unhydrolyzed extract samples from grey matter consumed about 0.03-0.30 ml — generally about 0.25 — of cerium sulphate per 10 mg dry substance, those from white matter 0-0.70 ml, generally below 0.10. As the samples for analysis from grey matter usually contained about 30 mg dry substance, those from white only about 10 mg, the influence exerted by reducing non-cerebroside substances on the value of hydrolyzed samples mostly was considerably greater for grey than for white matter.

*In cerebrosides*, isolated from cow brain in a phosphorus free state according to KLENK (103) and by paper chromatography (24) proved to contain galactose as the only hexose, the method yielded *the galactose value 21.9 %*, which agrees well with the theoretical figure (see p. 22). *Gangliosides* were conveniently isolated in a fairly pure state from brain of a child 2 months old. On subtracting the phospholipids (lecithin), amounting to about 25 %, the method yielded a value of 37 % for *hexose*, computed as galactose, in the remaining substance. It deserves mention as a comparison that in his purest ganglioside preparation KLENK found 43.5 % galactose.

*Standardizing* with and without hydrolysis in repeated experiments demonstrated a linear relationship between galactose and cerium sulphate quantities for amounts of galactose between 0.1-0.6 mg (others were not tested). 0.145 mg galactose or 0.109 mg glucose, respectively, caused the consumption of 1 ml of cerium sulphate. BRAND and SPERRY found the figure 0.147 for galactose.

*Error.* BRAND and SPERRY found the standard deviation for the determination on 1.2-1.4 mg samples of cerebroside, pure or contained in brain lipid extracts, to be  $\pm 1.9-2.3\%$ . The present author made only a few multiple determinations. The experiences from them did not deviate from those of BRAND and SPERRY.

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The statements of BRAND and SPERRY as to method, which on the materials in question most likely is specific for glycolipids, were confirmed. However, it requires relatively large quantities of test material and is in this respect inferior to orcinol methods. If the latter can be improved to completely exclude the disturbing influence of foreign substances (free hexoses, colorifying substances other than hexose), they will probably be preferable.

### Application of the Methods on Nerve Tissues

The determinations were routinely carried out on single samples for all the methods except the phosphorus analyses (total P and »KOH P») where determinations were made on two samples of each extract. As a rule a small portion of each extract was saved so that in doubtful cases additional determinations could be made. The average quantities of material required for each single determination were as follows:

	“Grey matter”, wet, mg.	“White matter”, wet, mg.
Dry substance, neuraminic acid	200	100
Total lipids, total choline	120	60
Lecithin-choline	150	100
Total phosphorus	20	15
Phosphorus acid soluble after KOH treatment	25	20
Serine	180	60
Ethanolamine	240	80
Inositol	300	150
Glycerol	140	70
Hexose	300	60
Cholesterol, free	10	2.5
Cholesterol, total	10	2.5
Quantity of fresh tissue required to do all the analyses	1695	720

As a rule it was impossible for reasons of time or with the quantity of material available to carry out all the analyses. As is suggested in the above discussions it is possible, however, to reduce the material requirements for some of the methods, e. g. for the disproportionately material-requiring determinations of inositol, hexose, choline and possibly colamine. By so doing *it would be possible to arrive at a total material requirement of less than 1 g grey and 0.5 g white matter, respectively.*

### Treatment of the Analysis Results

In order to make them conveniently comparable with the findings of earlier investigators the results have as a rule been multiplied by suitable factors (see discussion, p. 23) to make them valid for lipid groups. The values for total lipids and cholesterol require no such conversion. The average phospholipid molecular weight has been arbitrarily put at 775, the glycolipid molecular weight at 820. The »diglyceride» molecular weight is assumed to be 590 (i. e. containing fatty acids of the same molecular weight as do phospholipids), that of inositol phospholipids to be 848.

### Principles of Computation

*Calculated directly from the analysis value*

Galactose  $\times 4.55 =$  cerebrosides

Phosphorus  $\times 25 =$  phospholipids

Phosphorus, acid soluble after 1 N KOH treatment,  $\times 25 =$  »KOH-decomposable» phospholipids

Choline  $\times 6.4 =$  choline phospholipids

Choline, acid soluble after 1 N KOH treatment,  $\times 6.4 =$  lecithins

Glycerol  $\times 8.42 =$  glycerol as phospholipids

Ethanolamine-N  $\times 55 =$  ethanolamine phospholipids

Amino acid carboxyl-N  $\times 55 =$  serine phospholipids

Inositol  $\times 4.7 =$  »diphosphoinositide»

*Calculated by difference*

Phospholipids — choline phospholipids = cephalins

»KOH-decomposable» phospholipids — lecithins = »KOH-decomposable» cephalins (A)

Cephalins — cephalins A = cephalins B.

Choline phospholipids — lecithins = sphingomyelins

[Total glycerol — glycerol in »KOH-decomposable» phospholipids (assuming them to be monoglycerolipids)]  $\times$  6.41 = »diglycerides»

Total lipids — (phospholipids + cholesterol + cerebrosides) = unidentified fraction.

When the analysis is as complete as possible the results may conveniently be grouped as follows.

Total lipids

Phospholipids

Cholesterol free and total

Cerebrosides

Unidentified fraction

Individual phospholipids:

Lecithins

Cephalins A = »KOH-decomposable» cephalins

Cephalins B = »KOH-non-decomposable» cephalins

Sphingomyelins

»Diglycerides»

Ethanolamine phospholipids

Serine phospholipids

»Diphosphoinositide».

In studying the results of the analysis it should be kept in mind that the exact nature of the groups *Cephalins B* and »*Diglycerides*» is unknown (see pp. 43 and 47). As a matter of fact the former might well contain sphingolipids, the latter could be composed wholly or partly of triglycerides or diglycerophospholipids. If this were so the value for the latter fraction should rightly be corrected by multiplying it by about 1.41 or 1.31, respectively. If di- or triglyceride groups exist besides the phospholipid group the »Unidentified fraction» will be reduced by a corresponding quantity, if the »diglycerides» in reality are in combination with phosphoric acid the substance group is nothing else than a part of the cephalins, perhaps contained in a cardiolipin.

The individuals in »*Unidentified fraction*» are assumed, if they are not di- or triglycerides, to consist of such prestages or

decomposition products as must arise in the metabolism of the identified more complete lipids. Their nature was discussed in connection with the lipid classification on pp. 19 and 21. That many of them do occur is unquestionable, the problem is only to what extent. At present our knowledge of this subject is limited; all we can say is that they probably do not constitute a greater part of the total quantity of lipids.

Since only a limited number of analyses of some lipid groups have been carried out, the table is in individual cases often reduced. *Both free and total cholesterol were determined throughout but as the values in most cases were practically identical, i. e. all cholesterol was free, this group has been subdivided only in the cases when a significant quantity of bound cholesterol was present.* As the lipid groups calculated from difference have greater errors, the groups calculated directly have as a rule been reported in the tables as well. The names of the former groups have here been marked off from those of the latter by being placed more to the right of the margin.

#### Basis of Calculation

Since the water content is a relatively variable factor, at least in human clinical material where the mode of death (e. g. under blood stasis) and subsequent preparations (e. g. water irrigation during autopsy) vary with consequences difficult to control, calculation on a wet basis will often be somewhat misleading. Computation on a total solids basis yields a more constant result. The latter method has therefore been adopted for routine use in the present work. The greater part of the author's material, calculated in this manner, may be found in Appendix I.<sup>1</sup> With the aid of the figures given there the results may, if desired, be recalculated on the basis of any other suitably method. Values calculated on a total solids basis often do not give the clearest picture; sometimes it is possible to penetrate further with values on a wet basis, a total nonlipid solids basis, etc.

The relationships between the individual lipids become particularly lucid on a total lipid basis or a »total identified (essen-

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<sup>1</sup> The tables in the Appendix are numbered with Roman numerals, those in the text by Arabic ditto.



tial) lipid» basis, those of the phospholipids, especially, on a total phospholipid basis. Whenever in a special experiment some group remains unchanged, any changes in the others may become very apparent calculated on the basis of the former group. Lastly, in some cases it may be of interest to know the absolute contents in an entire organ.

Bases of computation of all these additional types were tested throughout by the present author, and when they yield something of importance the values obtained with their aid are given.

Statistical treatments were performed acc. to DAHLBERG (40).

### Summary

*For the application in analysis of lipids in nervous tissues of principles described in chapter I a number of micromethods developed during recent years are compared and contrasted. The procedure adopted for routine work in addition to dry substance determination includes determination of total lipids, phosphorus, KOH + mild acid releasable phosphorus, choline, KOH releasable choline, ethanalamine, amino acid, glycerol, hexose, inositol and cholesterol, in a total lipid chloroform reextract.*

*It is stated that the extract may contain non-lipid impurities but ordinarily in so small quantities that they do not significantly affect other than the total lipids, amino acid, and inositol methods, whose results therefore must be considered with some caution.*

*It is maintained that complete analysis supplies information as to the contents in a tissue of phospholipids, cholesterol, cerebrosides and any greater amounts of unidentified lipids (e. g. neutral fat), moreover as to the individual phospholipids lecithins, cephalins, phosphatidyl ethanalamine and serine, and sphingomyelins. — Applied to purified lipids and extracts from brain the methods have indicated the presence in small quantities of lipids other than the ones usually recognized in the nervous system. It would then be a case of phospholipids with difficultly releasable P without choline and phospholipids un-*

*usually rich in glycerol. — Certain facts suggest the presence in lipids of other bases than those known hitherto and then possibly someones partly codetermined in the determination of choline. — Some technical details are discussed and improvements described. The error of method for the separate methods is given and varies between  $\pm 1$  and 4 %. Possible means of further improving and developing the methods are given.*

## Chapter III

### On the Selectivity of Brain Lipid Determination by Fractionation with Organic Solvents

The author considered it desirable to test his methods on lipid fractions obtained from brain tissues with some of the fractionation procedures, by solvents, which formerly were much employed in lipid analysis. WEIL (189) had made use of such a method, arranged according to the well-known principles given by MACLEAN, and had carried out some interesting experiments on nervous tissue. WEIL's method was adopted for the present investigation.

**Experimental.** Pure cortical tissue (47 g) and pure white matter (71 g), respectively, were carefully dissected out from the brain of a 19 year old man, who had died in uremia. The tissues were cut into small pieces and ground in a mortar with 15 volumes of acetone. The mixture was allowed to stand for 24 hours with occasional stirring. The supernatant was drawn off on a BÜCHNER funnel (filtrate = acetone I extract). The residue was rapidly dried at 45° in a nitrogen current, ground in a mortar and exhaustively extracted with alcohol in a Soxhlet apparatus. The extract was evaporated under reduced pressure on a water bath until the first crystalline deposit appeared. Then the solution was precipitated with 10 volumes of acetone. When the deposit had settled well the supernatant was withdrawn through a BÜCHNER funnel (filtrate = acetone II extract). The precipitate was then repeatedly pestled with 100 volumes of alcohol during 24 hours. The fluid was drawn off through a BÜCHNER funnel (filtrate = alcohol extract). The residue was pestled with 100 volumes of ether for 24 hours (filtrate = ether extract). The residue was heated with pyridine to 45° for 15 minutes, rapidly cooled to room temperature, allowed to stand 10 minutes, supernatant removed (filtrate = pyridine extract). The residue was subjected to repeated heating with ethanol and chloroform dissolving everything except a negligible, coloured rest (filtrate = chloroform-ethanol extract).

The extracts thus obtained were analyzed, the first three being evaporated at 60° under reduced pressure and reextracted with chloroform before analysis.

**Results.** The results appear in table III. For the sake of comparison this also contains the corresponding results from *total* extracts of grey and white matter from the same brain obtained by the alcohol-ether + chloroform reextraction procedure routinely adopted by the author. The good agreement between the latter results and the sums of the fractions will at once be seen. Evidently the extraction method adopted by the present author by no means is inferior to WEIL's in effectively releasing the lipids from the tissue. It also seems to be more selective for lipids, for the first three of WEIL's fractions contained substances that could not be reextracted with hot chloroform. The same thing is suggested by the relatively greater unidentified fraction in white matter by WEIL's method. The other values for the sum of the lipids in the WEIL extracts are higher only as regards cephalins B, but since cephalins A simultaneously are more than as much lower *it may be asked whether cephalins B do not contain at least some cephalins A whose phosphorus contents by the treatment has been rendered non releasable by KOH.* (Cf. the similar effect of formalin treatment, p. 94). An apparent increase of »diglycerides», which ought to be the result of such an effect, is evident as regards white matter.

Regarding the individual fractions several facts deserve attention. The lipids of unknown nature — unidentified, cephalins B and »diglycerides» — all largely belong to the more easily soluble fractions. The *unidentified fraction in white matter* is an exception being almost as abundant in pyridine + chloroform-ethanol. Here it *may therefore perhaps contain sphingolipid-like substances without phosphorus or hexose. A triglyceride nature of the »diglycerides» (cf. p. 72) seems less likely* in view of their scarcity in the acetone I fraction (nil as regards grey matter!) where, as is the case with cholesterol, any triglycerides mainly would be found. From the values white matter may, however, contain a small amount of neutral fat. In support of the view above that part of cephalins A during preparation became KOH non-decomposable may be regarded the fact that the distribution of »diglycerides» on the separate fractions rather well parallels that of cephalins B.

The parallelism is not absolute, however. As a rule ›diglycerides‹ occur in relatively smaller absolute quantities than do cephalins B. This may to a great extent be due to the assumed lower molecular weight for the ›diglycerides‹ employed in the calculations and to the occurrence among cephalins of phospholipids poor in glycerol such as diphosphoinositide. This lipid, in which the P is wholly KOH releasable and which therefore is to be found among cephalins A, contains only  $\frac{1}{2}$  molecule of glycerol per atom of phosphorus. Since ›diglyceride‹ glycerol throughout (see p. 72)) has been calculated under the assumption that KOH decomposable phospholipids contain 1 molecule of glycerol per P atom, the presence of diphosphoinositide in a fraction entails an improper reduction of its ›diglyceride‹ glycerol and consequently the ›diglycerides‹ calculated on that basis (in some fractions possibly the reason for negative ›diglyceride‹ values in the table). Conversely, diglycerophospholipids or triglycerides are perhaps contained in the acetone fractions in which case the ›diglyceride‹ value would be raised. Owing to the many influencing factors it is thus very difficult at present to form a definite opinion as to the presence, if any, and the amounts in the native state of cephalins B and ›diglycerides‹. At least in part they may be artefacts (cf. also pp. 47 and 97). Evidently the values obtained in tissues by the author's routine method for cephalins B and ›diglycerides‹ reflect the balance of influences in various directions. Therefore, and due to their being calculated by difference (cephalins B by difference of two differences), it is not surprising that in the author's experiments these vary more than the other lipid groups and consequently the figures for them must be considered with some caution.

It is also of interest to compare the *occurrence of colamine and serine in grey matter in relation to that in white matter*. In the acetone extracts colamine is practically the only base revealable by ninhydrin. This should mean that the cephalins in these fractions are phosphatidyl colamine, i. e. in grey matter 6.5 %, in white 4.9 %. Colamine is present in considerable amounts in the alcohol fraction too. Assuming that  $\frac{2}{3}$  of the cephalins in the alcohol fraction are phosphatidyl colamine the total amount of this substance will be 8.1 % and 8.8 % in grey and white matter, respectively. Assuming the rest of the cephalins to be of serine type this very approximate computation suggests that *phosphatidyl serine occurs in greater quantities in white than in grey matter*. This subject will be discussed more in detail below (p. 120). So will some problems regarding the presence of inositol in lipid extracts. Here it is sufficient to mention that the greater part of the inositol extractible with lipids resists extraction with acetone followed by chloroform re-

extraction. Surprisingly great quantities are found in the alcohol fraction while the ether fraction contains 0.11 % and 0.09 % diphosphoinositide calculated on a wet basis in grey and white matter, respectively. As a comparison it may be noted that FOLCH's yield of pure diphosphoinositide from a similar ether extract was 0.05 % of fresh brain (66).

### Comparison with WEIL's Experiences

In fresh human brain WEIL 1929 (188) obtained the following total quantities of lipids:

		Acetone I	Acetone II	Alcohol	Ether	Pyridine	Rest	Total
Grey matter:	Weil	15.4	15.6	2.7	5.6	2.0	1.9	43.2
	Brante	18.9	12.4	5.6	2.7	1.6	1.1	42.3
White matter:	Weil	21.3	17.0	5.2	11.3	9.9	4.2	68.9
	Brante	21.2	11.2	11.8	3.0	11.0	5.9	64.1

The present author's results from non-reextracted fractions are given simultaneously (exception: Acetone II, white matter, which is a reextract).

Evidently the agreement between WEIL's and my values on the whole is rather good except for the alcohol and ether fractions where the values appear to be reversed. Possibly this may be due to the fact that the present author used 96 % alcohol while WEIL might have used absolute alcohol, possibly it is an effect of autolysis (see p. 87; WEIL's material was fresher) or the uremia.

WEIL also determined the P contents in some of the fractions. Expressed as phospholipids ( $P \times 25$ ) in % of dry tissue the results were:

		Acetone I	Other fractions	Total
Grey matter:	Weil	4.7	18.8	23.5
	Brante	5.8	13.2	19.0
White matter:	Weil	2.9	23.0	25.9
	Brante	2.4	22.2	24.6

It should be observed that BRANTE'S values are partly obtained from reextracts which may explain the lower contents in »other fractions» in grey matter.

With the exceptions mentioned, the fractions obtained by the present author are largely equivalent to WEIL'S. Apparently the latter author considers the contents of the fractions to be the following. Acetone: cholesterol + some phospholipids; alcohol: chiefly lecithins but also cerebroside and sphingomyelins; ether: chiefly cephalins; pyridine: chiefly cerebroside; residue: chiefly sphingomyelins. He is fully aware, therefore, that the *fractionation is incomplete*, viz. that perhaps 50 % of the phospholipids are lost in the acetone extracts and that the substance in the pyridine extract contains but 40 % of the galactose quantities to be found were it pure cerebroside. This agrees well with the present author's results. However, it was unexpected that *practically all the lecithin ends up outside the alcohol fraction*, and *merely about 50 % of the cephalins in the alcohol and ether fractions* while considerable amounts are contained in the pyridine extract and residue, or that *so large quantities of cerebroside pass over into the acetone extracts* (in grey matter most of them!) Results obtained by a method like WEIL'S are therefore often difficult to assess. A further discussion of this matter will be given in later chapters (IV and V).

### Summary

*The present author's routine methods were used to test the validity of a procedure for lipid determination (proposed by WEIL), based exclusively on fractionation with various organic solvents. It turned out that all the fractions were mixtures, and that some had an unexpected composition, very different from the one assumed by WEIL. Conclusions based on the relationships between and behaviour of such fractions must be judged with great care and some earlier experiences reconsidered in the light of the new knowledge.*

## Chapter IV

### The Effect of Autolysis on Lipids in Nerve Tissues

Since many of the materials the author planned to study could be obtained only some time after death the effect of autolysis under various conditions occurring in practice must be known if the results are to be properly assessed.

Earlier investigations (e. g. 164, 57) have as a rule demonstrated that in the nervous system the bulk of lipids both *in vivo* and *in vitro* are metabolized at a slow rate only. However, important displacements in the subgroups of the phospholipid group were thought conceivable in connexion with autolysis; they may be demonstrable only in pure grey or white matter; appropriate experiments were therefore carried out.

**Experimental.** Various materials from the central nervous system of cow were used. After suitable intervals they were frozen in »dry ice«, sectioned, and subjected to extraction, dialysis, etc.

In some experiments (cows I, except the samples in the third column, II-V) the material was taken from one of the hemispheres, the skull was closed, autolysis was allowed to go on for the stated duration, and a new sample was taken from the exactly corresponding place in the other hemisphere. In some cases the autolysis was carried out with sectioned tissue in closed glass jars (cows I, third column, IV), in others with sectioned tissues finely dispersed in certain salt solutions (cow VII-XI), sometimes with  $O_2$  supplied (cow X spleen nerves). In addition to 0.9 % NaCl (cow VII white matter) the more complex solutions of GÖTHLIN (77) and TASAKI (177) were used. The GÖTHLIN solution was slightly modified by substituting half the  $CaCl_2$  equivalents with the same number of  $MgCl_2$  equivalents and addition of 0.1 % glucose. The pH of the solutions employed was initially set at about 7 but this value was not checked subsequently. During the autolysis the liquids were carefully agitated several times. As a rule the dispersion was done in a given volume of the solution with subsequent pipetting with the same pipette to a number of dialysis tubes (cows VIII, IX-XI), in one case, however, (cow VII) directly in the tube in which instance a weighed amount of substance was poured into the solution in the tube. Of grey matter 150-200 mg/ml of solution was



used, of white matter 120-140. The external liquid was 10-20 volumes of the salt solution of which the tube contained a unit volume. In some cases (see tables) the salt solution was shaken with toluene before use, otherwise no sterilizing precautions were made.

When the contents of the dialysis tube was to be extracted it was poured into alcohol-ether in an extraction flask, after which the tube was carefully rinsed with additional alcohol-ether. The drying before reextraction of such extracts must be performed with several successive portions of ethanol but despite this the chloroform reextracts usually became somewhat cloudy.

**Results.** The results are given in detail in tables IV and V (cf. also table 3), among other things also to give the reader some idea of the reproducibility of the values obtained by the adopted procedure. The intention was throughout to make for each individual as identical as possible the materials to be analyzed before and after autolysis. Obviously, however, some sources of error must of necessity make themselves felt, e. g. in sampling from different hemispheres or in pipetting off a dispersion of sectioned tissues. It may similarly be difficult to transfer all the material from the analysis tube although this procedure always seemed to succeed. With the method of computation employed such errors would affect the values of all the lipid groups in a sample in a similar manner. The proportions between them should not be much influenced. Whenever such sources of error are suspected a computation based on the total amounts of analyzed lipids, or on some lipid that remains unaffected by the autolysis (e. g. cholesterol according to SPERRY (167)), would probably give a truer picture of what has taken place during the autolysis. In those cases when cholesterol on a dry substance basis has changed significantly during the autolysis error may be suspected.

If, with these reservations in mind, the results of fairly mild autolysis are studied, it is remarkable that, even with the generally adopted computation basis, well reproducible values are as a rule obtained in comparison to the initial figures. In general the deviations lie within the limits of the above reported errors of methods. It is also obvious that in themselves dilution of the tissues in salt solutions and storage in a dialysis tube do not necessarily cause appreciable tissue losses (see e. g. white matter cow VIII). On the other hand these factors seem

to entail an artificial increase of the unidentified fraction and sometimes of the glycerol value (cf. the circumstance mentioned above that often the extracts could not be made absolutely clear).

*Autolysis in undiluted tissues.* The experiments were planned so as to cover the different possibilities likely to be met in practice in taking samples from animals and man. It was technically impossible to obtain material from cows earlier than 20 minutes after death. Naturally, it is conceivable that at this time some changes might already have taken place in some lipid groups.

*At room temperature* the changes in the following up to 72 hours take place but slowly. Although the observations often are slightly variable the conclusion is warranted that lipids, phospholipids and cholesterol during the mentioned period of time do not change significantly, nor do cerebrosides except for a possible reduction in grey matter. Nor is there any manifest, consistently similar tendency to change in any of the individual lipid subgroups. If anything it would be a slight increase of choline phospholipids to the detriment of cephalins during the first hours. »Diglycerides» possibly increase slightly.

A reduction of KOH non-decomposable phospholipids in grey matter is the most striking event during *autolysis at 37°* (see also cow X table V). The general increase of the values in spinal marrow from cow XII during the autolysis is possibly attributable to an increased extractibility of the lipids due to the autolysis. If the values are converted into % of total identified lipids the alterations become negligible except for a small reduction of the sphingomyelins. The same conversion of the values for cow I (grey matter) yields similar results (see table 3).

*Autolysis in salt solutions* (table V). From the very beginning it must be emphasized that other solutions than those employed may give other results, that a physiological supply of O<sub>2</sub> and CO<sub>2</sub> was not ascertained and that sterile conditions usually did not prevail. In *white matter* a reduction of most of the lipid groups could be observed. Converted into % of total analyzed lipids most of the changes disappear, however, as regards cow VII. Therefore they may be considered as being

Table 3. *Lipids in Nerve Tissues During*

Calculation	in % of total identified lipid					
	Cow VI spinal white			Cow I cortex		
	Directly	5 h.	15 h.	Dir.	4 h. 20°	6 h. 37°
Cholesterol	23.8	23.7	27.0	16.4	16.7	16.8
Cerebrosides	29.4	31.0	27.2	19.1	13.6	15.1
Lecithins	9.2	8.4	9.6	16.7	18.6	21.6
Cephalins A	20.9	19.5	20.8	34.0	36.3	42.2
"      B	10.2	10.0	10.2	5.7	2.2	3.4
Sphingomyelins	6.5	5.8	4.6	8.1	12.6	1.0

caused mainly by the differences in pretreatment of the samples (only subdivided, dispersed in saline, and dispersed in saline contained in cellophane, respectively). As regards cow VIII the samples were treated identically apart from the duration of autolysis. In this case there obviously seems to be a slight decrease in phospholipids and cerebrosides. As far as phospholipids go this *decrease* is mainly due to the loss of *KOH non-decomposable phospholipids*. Also calculated on a cholesterol basis (assuming this lipid uninfluenced by autolysis, see p. 82) this is evident. For *grey matter* also the decrease in the mentioned fraction of phospholipids is the plainest and most constantly recurring change whether or not toluene was added. Here other phospholipid fractions too are strongly affected but in no definite direction. The excessive reduction in spleen nerve cow IX *may* perhaps be due to bacterial action. *In contradistinction to the phospholipids cholesterol and cerebrosides are practically unchanged.*

*In brief* the experiments here described demonstrate that autolysis in intact nerve tissues at room temperature has no significant effect on the lipid results as obtained by the author's methods. At a raised temperature (37°) and in a homogenized state the result with or without simultaneous dialysis will be a decreasing tendency in the phospholipids probably largely caused by losses of *KOH non-decomposable phospholipids*;

*Autolysis (see text p. 83 and 84)*

			weight units/w. u. cholesterol			
Cow VII central white			Cow VIII spinal white			
Dir.	Dir. in saline	6 h. 37°	Dir.	1 h.	15 h.	24 h.
26.5	25.4	24.6	1	1	1	1
27.7	24.6	25.1	1.39	1.29	1.17	1.27
7.0	} 39.2	10.3	0.33	0.37	0.32	0.30
28.2		30.5	0.93	0.87	0.88	0.93
} 10.7	} 10.8	1.7	0.40	0.42	0.37	0.29
		7.9	0.31	0.25	0.22	0.26

the losses in % being largest for grey matter. Some reduction in cerebrosides cannot be completely excluded but if any, it is small. The autolysis probably does not affect the cholesterol fraction.

It must be emphasized that these statements refer to normal tissues. Infected or in other respects morbid tissues may yield different results.

### Discussion

Although — owing to the small number of experiments — the found changes hardly can be considered definitely established regarding the values obtained by differences, it is interesting to relate them to the experiences of some recent investigators.

Recent papers by SPERRY and collaborators (167, 164) contain a fairly full discussion of the changes in cholesterol and phospholipids during brain autolysis. The reader is referred to them and the paper by BACKLIN (10) for details. Here will merely be discussed *what seems established at the present time*. SPERRY (167) points out the possibility of getting different results by autolysis in *intact* tissue sections as opposed to *minced* tissue. In rat brain incubated in 0.9 % NaCl for 24 hours the reduction in cholesterol and phospholipids was no greater than to make it attributable to mechanical phenomena, not autolysis

(167). Similarly, in guinea pig brains autolyzed *in situ* at room temperature for 6 hours, FAWAZ et al. (57) found no significant change in phospholipids. The present author's findings on intact tissue from cow are in complete agreement with SPERRY's and FAWAZ's. Recently (164), SPERRY reported that in *homogenates* of rat whole brain tissue incubated in saline-carbonate buffer at 37.5° a consistent decrease, averaging about 8 % in 4 hours' autolysis and increasing with time, resulted in the amount of phosphorus extractible with alcohol-ether. Various things indicated that the decrease was due to a cleavage of phospholipids (not merely a loss of non-lipid phosphorus) by a mechanism present in brain tissue (not by blood enzymes). The process was no more effective in young rats with active myelination than in adult ones. It was assumed to be slow or inoperative in living animals due to their known low rate of phospholipid turnover in adult brain. Results in all essentials similar to the latter ones of SPERRY's have been obtained by FRIES et al. (71) who found the phospholipid decrease to be 10-15 % in 4 hours.

The present author's results on homogenates in GÖTHLIN's solution point in the same direction. For white matter (cow VIII) the decrease in phospholipids is found already after 1 hour, reaching about 7 % after 15 hours and thereafter increasing only slightly. For grey matter the decrease is greater. However, it must be emphasized that sterile conditions seldom prevailed. Addition of toluene at least partly prevented the diminution in total phospholipids, but not the relative decrease in KOH non-decomposable phospholipids. If any appreciable effect of infection is disregarded the present author's results would suggest that *the phospholipid decrease observed by SPERRY and other investigators in brain homogenates during incubation mainly should be due to the KOH non-decomposable fraction*. The fact that it can be discovered in finely subdivided tissues and only there may depend on a liberation of some enzyme from a tissue structure (axons, nerve cells?) where it normally would contact the substrate in minute amounts only or not at all. In this connexion it is interesting to note that histological investigations into »degeneration *in vitro*» have shown (see CAJAL (35) that in excised nerves

kept in LOCKE's solution signs of degeneration develop in heat but not at  $0^{\circ}$  with and without oxygen first in the axon and much more rapidly than *in vivo*. Excised nerve cords kept in a humid chamber or an »indifferent liquid» exhibited no such changes. Even if these findings may be taken as being purely physical phenomena it is not impossible that they are a counterpart to the chemical experiences just described. They indicate the significance of dilution by salt solution.

All the results of lipid autolysis referred to up to now have been obtained in unfractionated total lipid extracts. The observed lipid changes are consistently very moderate considered as a whole. In contrast to this, however, there are the extensive autolysis changes in *intact* nervous tissue reported by WEIL (190) and BACKLIN (10) in using fractionated extraction. After autolysis for 24 hours of brain and spinal cord at  $37^{\circ}$  and in 0.9 % saline the former author found that not less than 60-75 % of the phosphorus which before autolysis came into the acetone or alcohol fraction had become water soluble. Unfortunately no data are given as to the extraction technique. In the light of the experiences discussed above the conclusion is warranted, however, that WEIL's results as regards phospholipids must be due to a change in extractibility rather than to true decomposition. Similar reasons may be the basis of BACKLIN's results. This latter author maintains that in a single experiment with whole rabbit brain (24 hours, room temperature) he found statistically significant changes in some lipid fractions. Among other things he found an increase in unsaturated phospholipids and a reduction in cholesterol, amounting to 55 and 25 %, respectively. His values for the quantity of the fractions are based on non specific oxidative determinations of fatty acids and cholesterol selectively isolated by solubility and hydrolysis procedures. No P determination was carried out. Since BACKLIN's experimental conditions during the autolysis in no way differed from those adopted in previously reported works, it is very difficult to believe that the extreme increase in BACKLIN's phospholipid fraction was paralleled by a corresponding increase in lipid phosphorus. The considerable reduction in cholesterol is, in the light of the disagreeing results with more specific methods, as difficult to believe in.

In the opinion of the present author BACKLIN's results are more suggestive of a *changed fractionability of the lipids*. The sum of the lipids was practically unaltered.

Among BACKLIN's experiences there was also a reduction in the cerebroside values amounting to almost 15 %. He emphasizes the good agreement of this finding with KIMMELSTIEL's (94) of a reduction of cerebroside galactose during autolysis in guinea pig brain. However, later than both these authors FAWAZ et al. (57) found, using a similar, but improved, procedure to KIMMELSTIEL's, that there was no change in lipid galactose during autolysis in guinea pig brain. They dismiss KIMMELSTIEL's changes as non significant, viz. as lying within the limits of his error of method. The present author's results on cow brains largely agree with those of FAWAZ et al. A possible small change in the glycolipids especially in grey matter may on closer examination turn out to be due to gangliosides. Thus, BACKLIN's finding of a change in the cerebroside probably is an artefact and due to similar reasons as the phospholipid and cholesterol changes.

A modified extractibility and fractionability caused by autolysis is interesting and may point to a breaking up of lipoprotein complexes, cation changes, fatty acid alterations or the like. Such changes do not influence and are not revealed by a procedure like BRANTE'S.

Lastly, the dialysis experiments permit some assumptions to be made *as regards contaminants*. In white matter dialysis up to 15 hours causes the disappearance of only 7 % of total lipid extractible P (but practically all inositol); in grey matter the loss may be greater but at most 24 % (cow IX). These reductions include such as are caused by autolysis, adsorption at cellophane tubing, altered lipid extractibility, etc. If we permit ourselves to ignore ineffectiveness of the dialysis caused by film formation or, respectively, adsorption of water soluble non-lipids to the lipids inside the tube, the results consequently indicate that in white matter practically all the phosphorus determined by the author's method is lipid bound; in grey matter at least 80 %.

### Summary

*Autolysis in intact healthy nerve tissues at room or body temperature for hours up to several days had only negligible effect on the lipid contents. (Some data to the contrary in the*

*literature are interpreted as being caused by autolytic breaking of lipid linkages with other substances, fatty acid changes, etc.).*

*A small reduction in phospholipids, as a percentage greatest in grey matter, on the other hand seems to set in when homogenized tissue is incubated in certain saline solutions. The loss chiefly applied to phospholipids containing »KOH non-releasable» P (generally looked upon as being sphingophospholipids).*



## Chapter V.

### The Action of Formalin Preservation on Nervous Tissue Lipids

Since long the means for preservation of pathological tissues has been formalin. Samples from many interesting diseases in the nervous system could be used for lipid determination but for untoward effects of the formalin treatment. It is known, that formalin does not lack a destroying action on tissues. As far as lipids go phospholipids are particularly affected. However, the knowledge is limited as to the possible extent of the formalin effect, its dependence on time, etc. Conceivably, these processes develop so characteristically that, knowing the duration of the formalin treatment, a definitive idea as to the magnitude of the error implied in the individual case could be gained. For these reasons the present author studied the discussed problems.

**Experimental.** Materials from adult cow and from man were investigated. In the former case white and grey matter were largely separated, cut into about 5 mm thick slices weighing  $\frac{1}{2}$ -1 g. and placed in separate jars with at least 10 volumes of 10 % formalin in distilled water. The human brains in 1-2 cm slices had been put into about 1-2 times their volume of 10 % formalin in tap water. The formalin solution was not changed during the experiment.

As regards cow and one human brain tissue samples for analysis had been taken also in a fresh state before the formalin treatment. Subsequently samples of pure grey or white matter for lipid analysis were taken at suitable intervals. Of white matter from man substance was taken a little way from the surface. The surface layer had a more yellowish appearance and was analyzed separately in one instance (white matter child I, after 200 days, »yellow part«).

Already after 9 and 12 hours, respectively, white and grey matter from cow were apparently satisfactorily fixed in the formalin. Naturally this was largely due to the thinness of the pieces.

Before sectioning on the microtome the lumps were wholly submerged

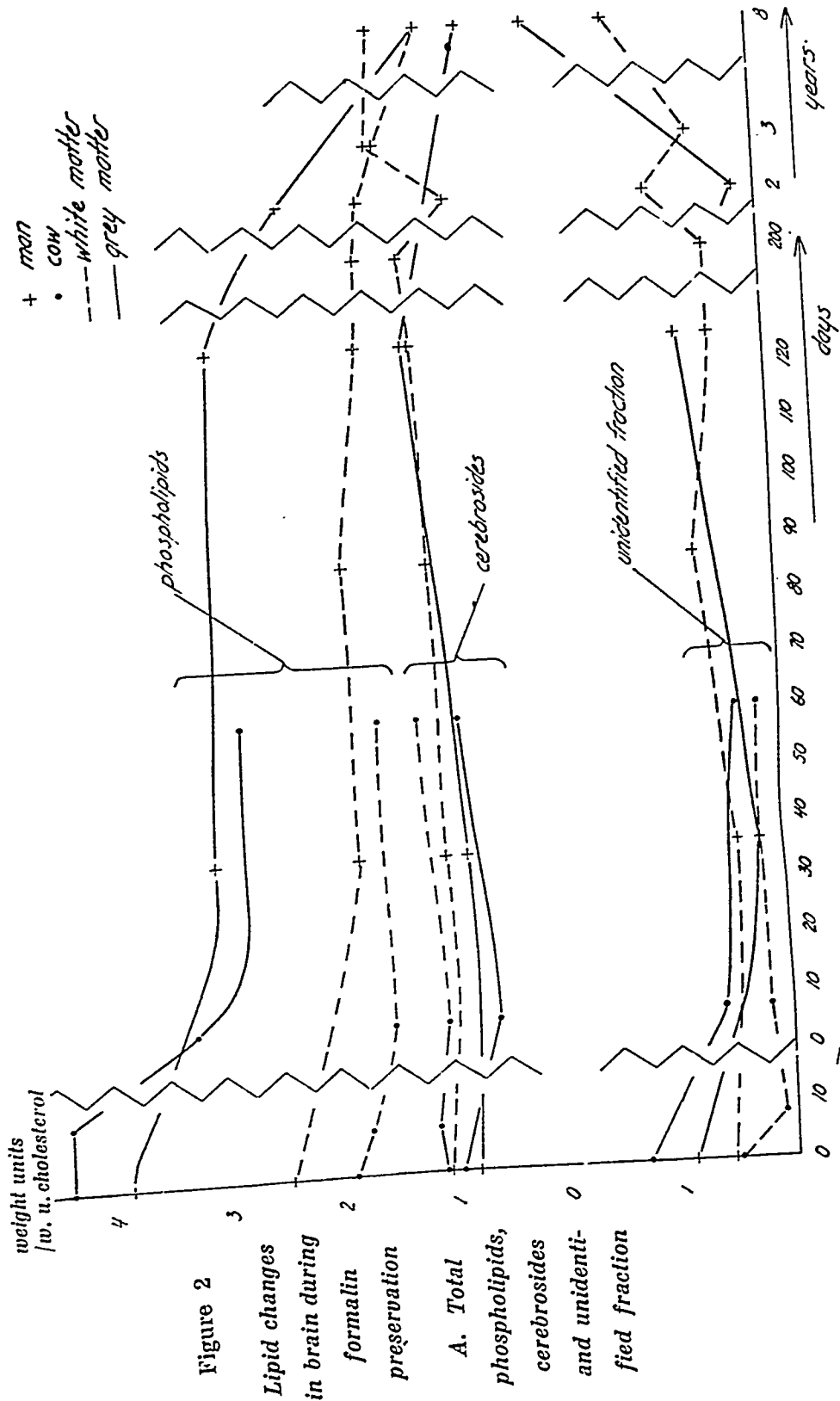
for a moment in distilled water, after which the surface water was blotted away with filter paper. It was sometimes found necessary to moisten the microtome table in order to make the substance adhere to it.

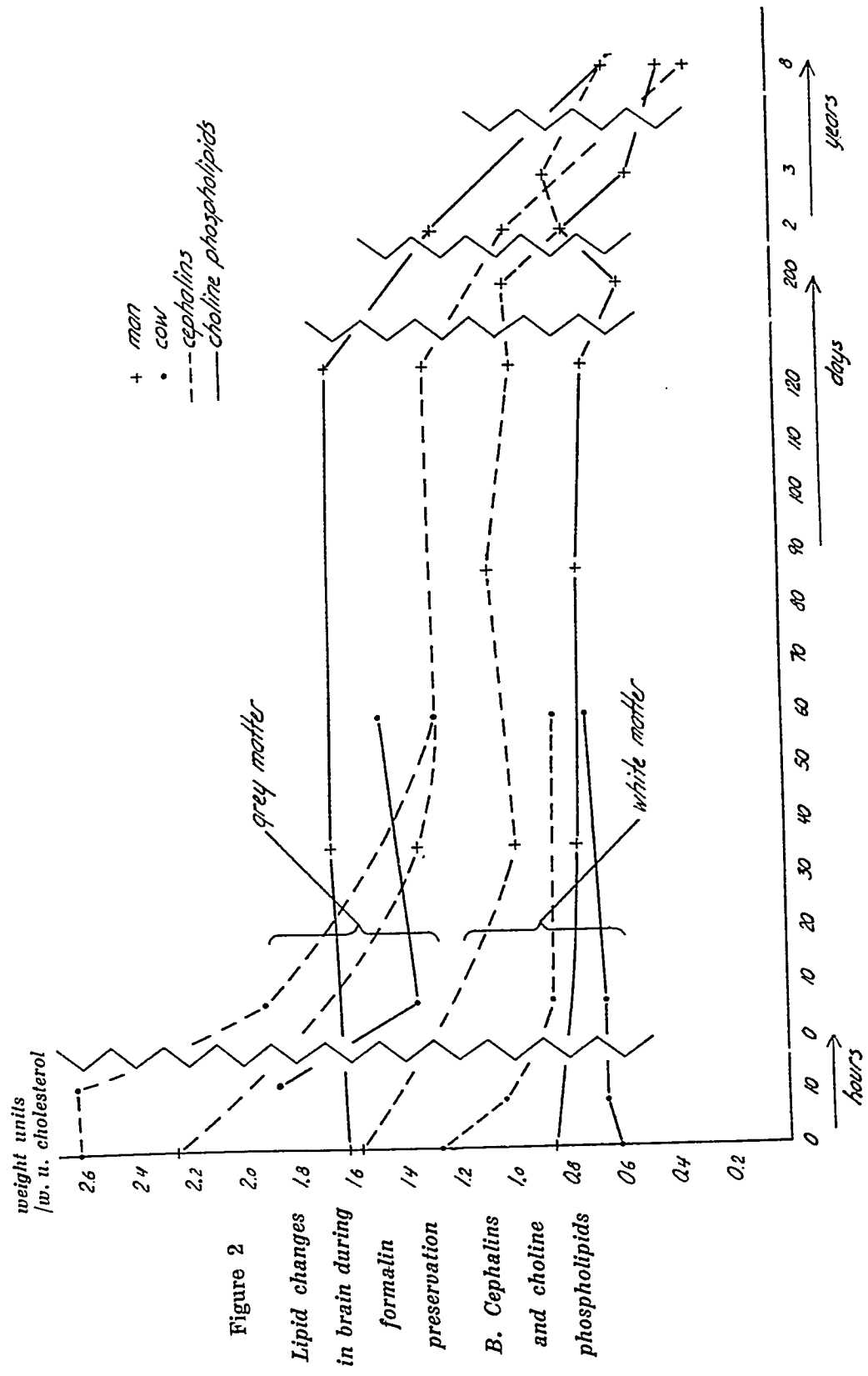
*Human material.* Child I, dead in edema glottidis. The histologically healthy brain was taken two days after death. Children II and III had no histologically demonstrable brain changes; corpus mamillare malacia was present in the otherwise histologically sound brain from Adult woman.

**Results.** The results are given in table VI. Before they are discussed some special considerations deserve attention. The compared samples are not altogether equivalent, not even when they come from the same brain, for they are taken from various places in it. Owing to the admixture of extra water, which probably could not be mixed into the substance quite uniformly, the determination of dry substance may have suffered from a greater error than usual. This of course influences the values computed on the basis of total solids.

The formalin treatment can certainly affect both proteins and extractives as well as lipids. The composition of the dry substance in a formalin treated sample therefore depends on how far and in which direction the effect has set in for the various groups of substances. Despite an absolute decrease a certain substance may therefore increase in relation to other solids. This makes it still more difficult to assess the value of results on a dry weight basis. The investigation would have been most easy to interpret if the absolute quantity of the various substances sought for was known in the sample before the beginning of the treatment. However, the author's experiments were unfortunately not so planned. In such experiments KIMMELSTIEL (95) could state that the quantity of cholesterol and cerebrosides in brain remained unchanged by formalin treatment, at least for the first 14 days. Computation of other substances on the basis of such unchanged substances can give a better picture of any changes in the former substances and has been adopted by the author (using cholesterol) alongside the routinely used method of calculation on total solids.

In table VI and figs. 2 A and B it may be seen that most important experiences as regards grey and white matter are of similar kind. At a very early stage (9-12 hours) all the lipids increase. The magnitude of this increase is what could be ex-





pected by a loss of the part of the solids made up of organic and inorganic extractives. Subsequently cholesterol and cerebrosides remain relatively unchanged or even increase, at least during moderately long durations; phospholipids on the other hand decrease already early, and this decrease continues down the years. The unidentified fraction decreases during the early stages and then increases rather steadily.

During the early stages the choline phospholipids begin to decrease only relatively late, and this also applies to lecithins as well as to sphingomyelins.

The reduction in cephalins is in evidence among those decomposable by KOH, while cephalins B in the beginning increase, even markedly. But this is only apparently so because the P in cephalins A is evidently to a certain extent rendered KOH non-releasable by the formalin treatment. This circumstance also entails a fairly parallel apparent increase of the »diglycerides».

### Discussion

Formalin effects similar to those reported here has been found by MLADENOVIC and LIEB (126), KIMMELSTIEL (95), WEIL (188), HALLIDAY (82), among others. These authors describe the reduction in phospholipids as well as the relative constancy of cholesterol and cerebrosides. WEIL is the only one who attempted to find out which phospholipids are reduced. With this in view he made use of the fractionation procedure described above (p. 76). The changes found by him were very similar for white and grey matter. The acetone I (»cholesterol») fractions consistently increased somewhat in quantity while acetone II in most cases decreased. In sum the other fractions decreased. This reduction was entirely caused by the ether and residual (= chloroform-ethanol) fractions which decreased in both grey and white matter in both cat and man while the alcohol and pyridine (»cerebroside») fractions were unchanged or even increased. Since the decrease of acetone II fraction largely may be suspected to be due to the loss of extractives, the greater part of the decrease in phospholipids can in all probability be attributed to the ether and chloroform-ethanol

fractions. According to the present author's survey over the contents of the WEIL fractions the combined phospholipid contents of the latter two fractions is about 85 and 70 %, respectively, of mostly serine containing cephalins in respectively grey and white matter. The decrease was most rapid in the beginning of the experimental period of up to 109 days.

Essentially the present author's data agree with those of WEIL. Thus, despite the fact that WEIL's experiences in part may be due to a change in the fractionability of the lipids (cf. p. 87) *it seems justified to at least largely attribute to cephalins, especially phosphatidyl serine, the reduction in phospholipids.*

Opinions have been divided as to the *mechanism of the decrease in phospholipids*. From the fluid used to fix brain for 14 days KIMMELSTIEL using hot alcohol could not extract more than insignificant amounts of phospholipids, not at all enough to correspond to the reduction in phospholipids in brain. So he believes that the latter is due to *a stronger fixing and lower releasability of the phospholipids* owing to the formalin treatment. HALLIDAY is of a similar opinion. She suspects that formalin treatment somehow changes lecithin chemically and bases the reduction in phospholipids in formalin preserved liver on this assumption. In support of this theory she points out that in a week or so pure lecithin diffuses out into a formalin solution; from the latter it cannot be extracted with alcohol-ether or chloroform. In experiments with liver MLADENOVIC and LIEB on the other hand find that *the phospholipids are decomposed by the formalin treatment*. WEIL is of the same opinion. Parallel with the loss in weight of some lipid fractions he found in the experiments described above that their P contents also lessened while P in the residue after extraction remained unchanged. The fixing fluid contained an amount of P corresponding to the loss in brain and increasing constantly with time. On formalin treatment carefully water extracted and denatured brain lost P at the same rate as not pretreated brain. WEIL concludes that formalin does not fix the lipids. On the contrary he maintains that the phosphoric acid part is liberated from the phospholipids — mainly the cephalin and sphingomyelin fractions — and is finally found in a water soluble combination. Between the beginning of

formalin preservation and this final stage there seems to be a stage in which at first are found intermediary products. These are not yet soluble in water but easily so in acetone and alcohol.

In the long run a release of the phosphorus part in phospholipids ought to lead to an increase of lipid extractible substance without P or hexose, i. e. of »unidentified fraction». There is such a tendency in the present author's experiments (after the initial decrease). Since there is no corresponding increase in glycerol the increase probably consists of fatty acids, i. e. *the lipids lose their glycerophosphoric acid part*. However, this effect is clearly in evidence only during the later stages. Possibly, therefore, the considerable initial reduction in phospholipids may be due to mostly other factors. KIMMELSTIEL's opinion as to the extraction of the phospholipids becoming increasingly difficult is offset both by WEIL's results and by the fact that in the present author's experiments (from white matter, cow, after 200 days) only insignificant amounts of additional substance could be extracted by continued extraction with methanol and chloroform after the routine extraction. A removal of undecomposed phospholipids from the tissue seems to be a more probable reason for *the reduction in the early stages*. If this is so and we are dealing with brain it is *not a matter of lecithin* as HALLIDAY suspects in respect of liver *but of phosphatidyl serine*. As is known this lipid is but slightly soluble in alcohol which may explain KIMMELSTIEL's inability to extract phospholipids from the fixing fluid.

Can phosphatidyl serine be released in 10 % formalin? The author tested his pure phospholipid preparations (see table II) finding that pure phosphatidyl serine unlike lecithin and phosphatidyl ethanolamine dissolved to a *clear* colloidal solution. The latter two formed cloudy solutions. In all cases the solution took 10-30 minutes. After exsiccator drying the lecithin and phosphatidyl ethanolamine could be dissolved in alcohol, phosphatidyl serine could not.

Therefore, it would be within the bounds of possibility that phosphatidyl serine is colloiddally released by formalin solution. The rapidity at which this then would take place in some of the author's experiments, e. g. white matter from cow, is so great however, that it may be asked if not the early reduction in phospholipids may be mostly an artefact and due to a passing away of chloroform extractible, water soluble non-lipids. LE BRETON (110) proved it probable that water soluble compounds of glycerophosphoric acid contaminate lipid extracts from many tissues. The present author found (p. 36) that free glycerophosphate may pass into chloroform together with lipids from brain, although the solution will be cloudy. In the formalin experiments with nervous tissue the chloroform extracts were always clear. The early, considerable decrease in the »unidentified fraction», however, suggests that it partly consist of water soluble non-lipids and that consequently a clear chloroform re-extract also contains some such contaminants. It is known that some dialysable phosphates accompany disphosphoinositide (66). However,

they can hardly amount to more than a few % of total lipid phosphorus. In the author's experiments glycerol decreased parallelly with phosphorus in the early stages of formalin treatment. This makes it probable that the disappeared P compounds mainly consist of glycerophosphates. Then they could be non-lipid in nature as in LE BRETON'S experience and this possibility is difficult to exclude completely. However, for the following reasons among others it seems to be of small importance quantitatively.

1). The results of autolysis contradict so significant an amount of non-lipid P in the chloroform reextracts as would be required by the reduction in phospholipids. 2). Grey matter from cow exhibited no measurable P reduction during the first 12 hours although the formalin had permeated the tissue. 3). WEIL'S results.

While it remains to prove early occurrence of phosphatidyl serine in the fixation fluid, largely in agreement with WEIL'S findings, the sequence, due regard being taken to all the facts mentioned, of the effects of formalin fixation on nervous tissue phospholipids may be the following. At an early stage a rapid outward diffusion sets in of cephalins, in the first place phosphatidyl serine, into the surrounding formalin solution. As the concentration in the latter increases the release diminishes. In addition a slow splitting off of the glycerophosphoric acid part of the phospholipids takes place from the very beginning and in the long run this leads to practically complete disappearance of all the phospholipids.

In view of the above *lipid analysis of formalin treated materials must be critically looked upon and regard taken to the duration of the treatment* and how it was carried out (e. g. exchange of solution, acidification of same, etc.). Under such circumstances it should be possible to draw valuable conclusions even when the material has been in formalin for a year or more. Very brief formalin treatment, for example in the form of some buffered saline solution, might even be developed into a suitable pretreatment before the lipid extraction to remove water soluble contaminants. Without stating the duration of the formalin treatment SMITH and MAIR (162) have adopted a similar principle.

An important thing to remember is that the *formalin treatment, even of short duration, affects the hydrolyzability of some previously KOH decomposable phospholipids* (see p. 94). This is a very interesting observation. It does in fact show that glycerophospholipids can be resistant to the hydrolytic action of mild KOH and acid treatment. There is nothing to prevent glycerophospholipids with similar properties from being



present already before the formalin treatment or, respectively, from being formed during the extraction procedure, i. e. the group cephalins B should already normally at least in part consist of glycerophospholipids. This is probable, especially when there simultaneously is present an approximately equivalent amount of »diglycerides». In white matter, which contains a large quantity of cephalins B and no significant simultaneous amount of »diglycerides», it is less likely.

A valuable piece of information obtained from the formalin experiments is that fresh tissues apparently do not contain a disturbing amount of free choline which would have been demonstrated by a reduction of the lecithin figure after the formalin treatment.

Finally, the author wishes to briefly discuss the »*diphosphoinositide*» results. As pointed out above the contents on a wet basis of this group is equalized as regards grey and white matter. This may indicate a formalin releasability and free diffusability of the *majority* of the chloroform extractible inositol, on which, indeed, the results are based. Other circumstances also suggest a free diffusability. Chloroform reextracts from fresh brain gave a considerable inositol value even without hydrolysis, amounting to about 80 % of the hydrolysis value for grey and to about 50 % of same for white matter. Since undecomposed lecithin and cephalins do not promote growth, this result must mean that the extract either contains free inositol or some unknown inositol compound of lipid nature which is water soluble at the same time as its inositol part is very easily released. While the latter alternative is improbable the knowledge of the tendency of polyols to form adsorption compounds (61) supports the former explanation. A similar substance may underlie the disappearance of inositol in dialysis (cf. the autolysis experiments table V). In fact, it renders strongly suspect as being mostly free inositol all the author's diphosphoinositide values which therefore have been put in quotation marks. But as it may be interesting to follow the chloroform extractible inositol in the nervous system they are included and computed in the ordinary way.

### Summary

*It is again demonstrated that formalin treatment entails no losses of cholesterol or cerebroside from tissues, at least not during the first year or years, but that their phospholipid contents are reduced. During the first days the phospholipids disappear rapidly; during the following weeks the reduction*

rate successively diminishes to a low but constant level so that after some years there may be practically no phospholipids left. Lipid analyses of materials preserved in formalin must be considered in this light; their interpretation is difficult for preparations more than a year old.

The initial rapid reduction in phospholipids is one of cephalins, possibly phosphatidyl serine; later a slow, continuous decomposition of all the phospholipids into fatty acid and glycerophosphoric acid parts becomes apparent.

A special effect of formalin treatment is its rendering P in part of the glycerol phospholipids non-releasable by mild KOH and acid treatment acc. to SCHMIDT *et al.* (148).

## Part II

# Topical Distribution of Lipids in the Nervous System

## Outline

As was mentioned by way of introduction a main purpose of the author was to attempt to enrich the knowledge of the localization of the various lipids in structurally different parts of the nervous system. These matters have earlier been studied quite extensively. Of necessity chemical investigations have as a rule been carried out by means of macromethods, excluding many materials of interest from the possibility of being studied. Examination by histological methods, on the other hand, have often suffered from unspecificity or difficulties in interpretation owing to inadequate knowledge of the reaction mechanisms. With the aid of the micro-methods given in Chapter II it was possible to proceed further by chemical means. However, as yet it probably is impossible to isolate homogeneous types of cells and, even less so, smaller parts of cells for such a study. A method like that of CASPERS-SON'S for nucleic acids and proteins has not been reported for lipids. Things being as they are it is necessary to select materials where the mixture of cell types and structures is as in-complex as possible and then compare the analytic results with those from other materials containing one or more other cellular constituents. By investigating many dissimilar, relatively simple materials, it would be possible by way of rejection to get a more exact idea of the lipid contents in homogeneous cells or structures. This principle has long been in vogue and, availing himself of the resources open to him, the author has endeavoured to apply it as much as possible. Of course, many materials exist other than the ones studied hitherto which might

have afforded valuable information, e. g. the axons in giant nerve fibres, electric organs, brain lipidoses but as yet it has been necessary to leave them aside.

There hardly exist any types of animal cells without lipids. Cellular membranes, particularly, always contain lipids but also other cellular structures such as mitochondria, Golgi apparatus, etc., as well as the nucleus of the cell have been proved to contain lipids. In this respect the cells in nerve tissues are no exception. Both those of mesodermal and those of ectodermal origin have been demonstrated to contain lipids under physiological or pathological conditions. Lipids can even occur outside (and consequently between) the cells. The nervous system really holds a unique position in as much as in higher animals it is the tissue richest in so called essential lipids. It is well known that this is due to the high lipid contents in the myelin sheaths. Where these sheaths occur in great numbers they become the main factor determining the lipid pattern resulting in chemical analysis. When the not unimportant lipid accumulations in other parts of the nervous tissue than myelin sheaths is to be studied it is therefore necessary to avoid admixture of the latter.

To separate the tissue into pure myelinated (generally »white matter») and myelin-free (generally »grey matter») portions is not always easy or possible. The possibility closest at hand is to study brain cortex and, respectively, nerve nuclei and marrow separately, in which process sources of error from contamination of cortical grey matter by marrow radiations must be kept in mind. Heeding WEIL's advice (187) one should however, obtain a satisfactory separation. The »pure» grey and white matter obtained by such macroscopical separation are, however, by no means uniform. In addition to the neurites with their sheaths and axons white matter also contains glial elements of varying types and numbers and, of course, a certain amount of blood vessels with surrounding membranes. The vessels, and this also applies to cell body components, are even more important in grey matter which otherwise for the most part consists of nerve and glia cytoplasmic branches, together forming a closely knit filty tissue. A study of the arachnoid or of the meningeoma should give data, use-

ful for correction, on the lipid contents in, especially, connective tissue; the same information as regards the glia should be obtained by analysis of various glioma, especially more mature types, in doing which it must be remembered, however, that the chemical composition of tumour cells can differ considerably from that of their normal parent cells. This is also true in drawing conclusions as to the glial chemistry of cases with pathologically increased glia, e. g. diffuse sclerosis. The latter and certain other demyelination conditions in brain marrow are otherwise profitable objects for study in so far as at best they may exhibit a selective disappearance of the myelin sheaths in otherwise relatively unchanged tissue. During some developmental stages the brain contains similar materials since then the formation of myelin sheaths has not yet started. The formation of axons similarly should be examined at still earlier stages. They have also been studied in nerves poor in or free from myelin such as sympathetic and evertbrate nerves, where the neurites chiefly consist of the axon part. Of interest in the study of the lipid distribution on axon, myelin sheath and surrounding tissues may also be the quite complex changes taking place during nervous degeneration and regeneration, when the various histological structures alter at different rates. The nerve cells themselves are difficult to attack separately. Ganglioneuromas are rare and the nerve cells in them diluted with glia, so probably they would not be of much help. Analysis of individual nervous centres, different developmental stages of the cortex, etc. where various types of cells are represented might possibly yield some findings.

The present author has worked along lines similar to those outlined above. The investigations in this study will in the following be treated separately in the form of completed experiments which in themselves are considered of value and the validity of the results will be discussed from various aspects. The findings gleaned from them of importance for the author's main problem will then be dealt with in a final summarizing chapter where an attempted picture of the lipid distribution in the nervous tissue, based on experiences of the present author and earlier investigators with chemical and histological methods will be given.

Earlier reports, which are very numerous as regards several of the investigated problems, were thought to require a fairly full review — something apparently only incompletely done so far. In each chapter, therefore, a detailed discussion of earlier results, to a great extent recalculated to make them comparable to my own, is included. In cases of divergencies between findings possible methodological reasons are discussed.

## Chapter VI

### Distribution of Lipids in "Grey" and "White" Matter from Different Parts of Adult Human and Animal Nervous System

For the study in question the following different types of tissues were selected:

»*Grey matter*». Cortex cerebri, some subcortical nervous centres, symphatetic ganglia and nerves (interganglionic parts of truncus symphiticus, spleen nerves, nervus vertebralis), retina, papilla nervi optici (distally of lamina cribrosa), ventral cord and claw nerve of invertebrate.

»*White matter*». From corpus callosum, hemisphere (centrum semiovale) and spinal cord, nervus opticus, intradural nerve roots, peripheral nerve trunks.

In interpreting results from these tissues the following must in the first place be kept in mind as regards their histological composition.

*Cortex* preparations from adults always contain — even when, as in the present investigation, they are taken from the parts nearest the surface where the vertical myelin-fibre radiations largely have ceased — a certain admixture of tangential layers of myelinated nerve fibers. The types and amounts of the cells and the occurrence of myelin sheaths and vessels vary in different parts of the brain cortex. The lipids in various types of cortex were therefore studied. According to v. ECONOMO (47) in man grey cells on an average constitute 4 % of the total volume of the brain cortex (value uncertain acc. to AGDUHR (1)); the volume of the capillaries about 1.2 % (113). However, the total volume of blood and vessels probably may be higher; for the whole brain DONALDSON (44) puts it at about 6-8 % and the cortex is richest in vessels. It was difficult to find any quantitative data as regards the glia. DONALDSON estimated it at 2-4 % of the total brain volume but it probably

occurs most abundantly in grey matter. In man this latter substance takes up about 50 % of the total volume and consequently the glia in the cortex should amount to less than 8 % (if DONALDSON's data are correct). The axon-dendrite meshwork, would then amount to 75-80 % of the total cortical volume of which probably < 5 % contains stainable myelin sheaths. Similar conditions prevail in subcortical centres.

Judging by the consistency alone *sympathetic ganglia* are rich in supportive tissue of both specific (similar to glia) and aspecific (ordinary connective tissue) type. Consequently the true nervous elements are fairly dilute in these ganglia. The nerve cells are slightly different from those in the cortex, they are enveloped in a close meshwork of nerve fibres containing very few myelin sheaths. The interganglionic parts of *truncus sympathicus* also appear to be rich in connective tissue; their nerve fibres consist both of such poor in myelin sheaths and quite a number rich in myelin. *N. vagus* shows a similarly mixed but still myelinricher fibre picture. The fibres of *nervus vertebralis* are possibly all non-myelinated. FALK (56) has stated that the *spleen nerve* of cow is practically free from myelinated fibres. (In cat spleen nerve the latter fibre type is stated to make up 5 % of all fibres (185)). Its unmyelinated fibres are somewhat different to that of other »myelinfree» nerve fibres, of a more primitive type (173). Here the epineurium was easy to remove, but the interfibrillar connective tissue on histological examination certainly still exceeded 50 % of the volume.

Regarded as an example of myelin-free nervous tissue the *retina* is indeed anything but homogeneous. In addition to the quantitatively less important axon layer it also contains lots of supportive, nerve and sensory cells. From the point of view of homogeneity *papilla nerve optici* contains more pure axonic tissue; however, there is some admixture of vessels and supportive tissue. The *nervous system of the lobster*, finally, is nowadays considered to contain thin axon sheaths (151) with some but small amounts of myelin. According to YOUNG (200) the contents of connective tissue in the claw nerve is considerable. Probably one would not be far out by assuming that all the above mentioned materials from parts of the peripheral nervous system contain at least 50 % supportive tissues.



The various kinds of *white matter* differ histologically by the larger or smaller amount of tissue surrounding the nerve fibres, viz. glia, Schwann cells, connective tissue, fat tissue, blood vessels, and by different relative amounts of myelin sheaths as opposed to axons. White cerebrospinal matter contains almost unmixed nerve fibre tissue while peripheral nerves may be heavily diluted with connective and fat tissue. Sometimes the greater part of this can to a large extent be effectively removed, as e.g. by pulling off the epineurium and perineurium of nervus ischiadicus of rabbit, but the macroscopically inaccessible endoneural elements still comprise up to 50 % of the nerve (DE ROBERTIS (42)). The relative volume ratio axon/myelin sheaths varies considerably in various types of fibres and possibly also in different parts of the nervous system; in average it is given as  $\frac{1}{1}$ , by DONALDSON and HOKE, about  $\frac{1}{4}$  by ARNELL, both figures regarding spinal nerves and the latter, referring to a native state, probably the truest one. In the central nervous system and vegetative nerves owing to increased dilution with non-myelinated axons and short, slightly myelinated ones the average ratio will tend to higher values.

The author will return to the finer structural details in chapter XI.

### Experimental

The human material comprises individuals, 10 years or more of age and with healthy brains, in Chapter II; sympathetic chain came from sympathectomized hypertonics. The animals employed, all fresh adults, were killed without narcosis of any kind (cow and horse were shot, rabbit injected with air in vein of ear, rat was decapitated, lobster chilled until start of ice formation). The material was excised as soon as possible after death, immediately chilled in 'dry ice', and then stored at  $-18^{\circ}$  until analysis could take place (within a day for two). The time taken before the nerve tissues were excised could vary from 5 minutes for rat to 20 min.-2 hours for cow. The lobsters (12) were kept at low temperature all the time, the nervous material was transferred directly to frozen vessels. Papilla nervi optici was excised distally of lamina cribrosa from 40 cow eyes.

All preparations were carried out without the use of magnification. Connective tissue, vessels and any other undesirables were meticulously removed from the nervous material. Where nothing is said to the contrary brain cortex of larger animals was routinely taken from the greater portion of the medial surface of the hemisphere (where most cortical types are represented) by peeling off a layer about 1 mm thick. Cortex from rat was always collected from 4-5 individuals for each analysis.

As regards the other nerve cell materials also contamination with white matter was as far as possible avoided, and white matter was similarly taken without admixture with grey. White hemispherical matter was taken from centrum semiovale; the lateral columns from spinal marrow; the membranes were removed from peripheral nerves, an easy procedure in rabbit, less so in cow. The rabbit nerves are the normal ones in the degeneration experiments (chapter IX).

The different cortical tissue types in table VII were taken from the brain of a 12 year old subject from the following places selected according to v. ECONOMO's well-known scheme: frontal type from gyrus frontalis superior; agranular type from gyrus praecentralis; polar type from gyri around bulbus olfactorius; granular type around fissura calcarina. Seemingly pure grey matter was taken from caput nuclei caudati after removal of the membrane towards the ventricle. As those above the hypothalamus preparation also appeared to become pure white, owing to its light colour, thalamus probably was admixed with some white matter.

### Results

The results appear in tables VIII<sup>1</sup> and VIII<sup>II</sup>. For some of them the standard deviation is computed despite the comparatively few individual determinations. Other multiple determinations agreed within equal limits of error. On the part of rabbit the dispersion is small, probably because this material was most uniform. For man the values are more dispersed which by no means is surprising in view of the great differences in age of the individuals, kind of disease or mode of death, storing time after death, etc.

*The agreement between brains of various mammals in lipid contents on a dry weight basis is striking both as regards cortex and marrow.*

**Grey matter.** Commencing with a relatively simple structure, the *nervous system of lobster* contains but a small proportion of lipids in the strongly watery tissue. Centrally and peripherically the composition is much the same. *Phospholipids predominate markedly over cholesterol and cerebrosides* which occur in minute quantities only. While the strong and in all respects typical colour reaction according to SCHOENHEIMER and SPERRY renders doubtless the *presence of a sterol and with all probability cholesterol*, the cerebrosides were indicated only by a reduction value fairly low for the determination in question. Before their presence can be considered absolutely established further examinations should be carried out by more specific

methods. Apparently the phospholipids mainly consist of »choline» phospholipids (80-90 %) while the contents of cephalins are low. The base in the former may be another than choline for its reineckate precipitate seemed more finely grained and heavy than usual, of typical colour, however. Unfortunately the experiments to determine KOH releasable P miscarried, but the value for glycerol as phospholipids together with the choline values suggest that the occurrence, if any, of KOH non decomposable phospholipids, and thus of sphingomyelins, must be insignificant. Most (80 %) of the phospholipids consists of a substance classifiable as lecithin. Neutral fat is negligible or absent; so are cholesteryl esters.

As regards *mammalian peripheral nerve materials*, poor in myelin, things are somewhat different. Throughout their phospholipids contain in addition to lecithins considerable amounts of cephalins and sphingomyelins. Choline phospholipids here make up 41 % (interganglionic parts of sympathetic chain) to 52 % (n. vertebralis); lecithins are somewhat more plentiful than sphingomyelins. There also seems to be a small quantity of cephalins. B. Repeatedly obtained considerable reduction values after correction for free hexose show that *glycolipids* certainly are present and in approximately the same amount as cholesterol. Small amounts of neutral fat may be present; cholesteryl esters are absent.

Totally the lipids in the axon materials discussed constitute only a relatively small part of total solids, viz. as a rule approximately 15-20 %. Much the greater part of the rest is no doubt proteins.

In respect of the lipid contents *sympathetic ganglia* do not significantly diverge from the materials free from nerve cells; *retina* as a whole as well as *pap. n. optici* are relatively richer in phospholipids, for the rest they agree.

Relative to those tissues hitherto described *brain cortex* exhibits a much higher lipid level, lipids constituting about 35 % of total solids. Here also the phospholipids predominate while cholesterol and cerebrosides, occurring in about equal quantities, together weigh only half as much. A good 40 % of the phospholipids are choline phospholipids, and  $\frac{1}{4}$ - $\frac{1}{3}$  of these latter are sphingomyelins (in cow rather more). About

80-90 % of the cephalins are KOH decomposable. The cephalins are made up of about 75 % ethanolamine phospholipid (table 4), and most of the remaining 25 % probably is phosphatidyl serine. (The high amino acid value obtained in the analysis probably is due to contamination, cf. p. 32.) Then there also is a small quantity of »diphosphoinositide», probably much less than is indicated by my value for chloroform extractible inositol. Significant amounts of esterified cholesterol are absent; the presence of small amounts of neutral fats cannot be excluded.

The analyses of the *various cortical tissue types and basal ganglia* in table VII came early in my series of investigations. As I do not consider the varying cerebroside values quite reliable I have refrained from drawing any conclusions from them. Other results show that the *lipid composition does not vary considerably with the type of cortex*, the apparently lower cephalin contents in polar type possibly excepted. The lipids in nucleus caudatus (in this case, as in other human brains and cow brains analyzed) and hypothalamus grey scarcely differ from those in cortex, while the relatively high cerebroside and cholesterol values in thalamus may indicate some admixture of white matter — indicated already macroscopically by the light colouring of the preparation.

**White matter.** The fibres in *spinal marrow contained the greatest proportion of lipids*, or not less than  $\frac{3}{4}$  of total solids. Here also the phospholipid group was predominant and weighed almost as much as cholesterol and cerebroside together. Of the two latter the cerebroside were somewhat more plentiful. Choline phospholipids made up about 35 % of total phospholipids, lecithins and sphingomyelins were about equal in amount. 75-80 % of the cephalins are KOH decomposable. »Diglycerides» and therefore possibly neutral fats seem to be present in small amounts in rabbit but absent in the other animals; cholesteryl esters are absent throughout.

Both *towards the brain and towards the periphery*, as reckoned from the spinal marrow, *the lipid contents of white matter decreases*. The proportions among the individual lipids, however, are largely retained. Apparently conditions within the choline phospholipid group are an important exception

Table 4. *Composition of Cephalins in Grey and White Matter*  
(% fresh tissue)

S u b j e c t		Total ce- phalins (=phospho- lipids—cho- line phos- pholipids)	Ethanol- amine cephalins	Phosphatidyl serine	
				By differ- ence	By amino acid deter- mination
Grey matter	Human				
	11 years old:				
	Mixed cortex	1.95	1.43	0.52	1.42
	N. caudatus	1.75	1.31	0.44	1.86
»	Average	1.85	1.37	0.48	1.64
White matter (brain)	Cow I	5.94	2.65	3.29	4.59
	" II	6.60	2.42	4.18	4.12
	Human				
	11 years old	6.24	2.67	3.57	3.51
	Human				
	48 years old	6.04	2.43	3.61	—
»	Average	6.21	2.53	3.67	—

because *peripherally from the brain the sphingomyelin group increases steadily in proportion to the lecithins*; in peripheral nerves the ratio between these groups is about 1.8/1, in brain white about 0.7/1. The low cerebroside values in intradural nerve roots may be accidental, for in other determinations on nerve roots the author has obtained cerebroside results of the same magnitude as the cholesterol values. (The determinations on nerve roots from various segments and different individuals yielded fairly diverging results and therefore require further investigation.) *Corpus callosum* (case 2 years, table IX) gave results which largely *agreed well with the values from centrum semiovale*.

Experiments to determine the distribution of ethanolamine phospholipids and serine phospholipids demonstrated that the latter are predominant, amounting to about 60 % as against 40 % of the former of total cephalins (table 4). The »diphosphoinositide» values are of the same magnitude as in grey matter; peripherally, however, they increase slightly.

## Discussion

### I. Earlier experiences

*Vertebrates.* First will be discussed the correlation with recent results obtained along similar lines. While the present work was being written up there appeared some reports by JOHNSON et al. (88, 89) who in studying lipids have adopted methods in many respects similar to those of the present author (same cerebroside, cholesterol, lecithin and KOH releasable P methods). In an ethanol and ether extract they determined cerebroside directly, phospholipids in a precipitate through acetone and  $MgCl_2$ , and cholesterol in the supernatant acetone solution. They carried out a few investigations on lipids in grey and white matter from normal brains. Some of their results, expressed in mg/100 mg fresh tissue, together with some of the present author's results, recalculated on the same basis, appear in table 5.

The results exhibit some typical differences. In *grey matter* cholesterol and cerebroside throughout lie on a higher level in the determinations of JOHNSON et al. The underlying reason may be a less successful separation of grey and white matter. The other values are not significantly different. More marked deviations as regards *white matter* from brain are present for KOH non-decomposable phospholipid values which throughout are higher in JOHNSON et al.'s material, while the values for KOH decomposable phospholipids are correspondingly or even more so lower. The question immediately arises if the acetone- $MgCl_2$  precipitation may not have a similar effect on the hydrolyzability of P from some glycerophospholipids to, for example, formalin treatment (see p. 97)? Lastly, the values for the various lipid groups in *nervus ischiadicus* of rabbit in JOHNSON et al.'s experiments are much lower than BRANTE'S all along the line, while the proportions between them (with the exception of KOH decomposable and non-decomposable phospholipids) are much the same. The removal of the epineurium, which was maximum in BRANTE'S cases, may have been imperfect as performed by JOHNSON et al.

SCHMIDT et al. (148), who were the first to make use of

Table 5. *Lipids in Grey and White Matter acc. to Johnson et al. (88, 89) and Brante (% wet tissue)*

		Cortex							
		Human		Cow	Rabbit	Rat	Dog	Cat	
		Brante	Johnson et al. 1	Johnson et al. 2	Brante	Brante	Brante	Johnson et al. (2)**	Johnson et al. (2)**
Total lipids	»	5.30	5.1*	5.3*	5.89	6.63	7.05	7.04*	7.01*
Phospholipids	»	3.08	3.12	3.48	3.54	3.76	4.45	4.20	4.29
Cholesterol	»	0.77	1.00	0.97	0.82	0.88	0.93	1.33	1.25
Cerebrosides	»	0.63	1.20	0.63	1.20	1.09	1.24	1.52	1.48
Unidentified	»	0.82	—	—	0.33	0.90	0.41	—	—
Lecithins	»	1.10	0.61	1.16	0.96	1.32	1.53	1.00	(1.35)
Cephalins A	»	1.40	2.21	1.77	1.90	1.85	2.42	2.27	(1.94)
» B	»	0.30	0.30	0.55	0.25	0.22	0.12	0.93	0.71
Sphingomyelins	»	0.27			0.41	0.35	0.38		

		Brain white matter						Nervus ischiadicus	
		Human		Cow	Dog	Cat		Rabbit	
		Brante	Johnson et al. 1	Johnson et al. 2	Brante	Johnson et al. (2)**	Johnson et al. (2)**	Brante	Johnson et al. (3)**
Total lipids	»	17.9	14.85*	14.78*	20.0	20.7*	17.58*	19.8	11.9*
Phospholipids	»	7.73	6.24	6.80	8.65	8.47	7.96	9.76	5.94
Cholesterol	»	4.02	4.00	3.84	4.23	5.13	4.53	4.07	3.06
Cerebrosides	»	4.67	4.61	4.14	5.30	7.11	5.10	3.85	2.93
Unidentified	»	1.48	—	—	1.83	—	—	2.12	—
Lecithins	»	1.66	0.90	1.49	1.54	1.64	1.36	1.27	0.81
Cephalins A	»	4.11	2.60	1.80	4.86	3.17	2.59	4.74	2.26
» B	»	0.87	2.74	3.50	1.04	3.67	3.78	1.45	2.86
Sphingomyelins	»	1.08			1.20			2.30	

\* Sum of essential lipids

\*\* In brackets number of cases analyzed

the different decomposability of the phospholipids, reported the following results ( $P \times 25$ ; % fresh tissue):

	Lecithins + cephalins A	Céphalins B + sphingomyelins
Grey matter, ox brain	3.95	0.30
White » , »	7.38	2.33
Ischiadicus, rat (5)	6.50	1.73
» , cat (3)	4.43	3.73

These results, obtained in a chloroform-methanol reextract without any precipitation treatment, agree more closely with those of the present author as regards the relative magnitude of the group of KOH non-decomposable phospholipids.

Still earlier investigators who have analyzed grey and white matter separately have generally adopted very different methods by which the phospholipids have not been divided into the same subgroups as the present author's.

RANDALL (141) has studied alcohol-ether extracts of brain materials from normal and psychotic subjects. As in my own investigations various cortical regions (frontal and parietal) and nucleus caudatus agreed mutually as regards the lipid contents and so did also different »white matters» (corona radiata, frontal white, parietal white). The brain stem values, on the other hand, seemed to diverge away from those of pure white matter towards the grey matter values, the thalamus values away from pure grey matter values towards white matter values; the two tendencies to deviate might well be due to admixture of grey and white matter, respectively, which is likely to occur in the regions in question. The following means on a dry weight basis for respectively pure grey and white matter can be extracted: total lipids (BLOOR's oxidative technique) 32.7 % and 56.0 %; phospholipids (by P determination) 25.5 % and 30.2 %; cholesterol (BLOOR's colorimetric procedure) 6.29 % and 14.4 % (of which little or none in esterified condition). Cerebrosides were not determined but computing by differences they would be practically absent in the cortex and amount to about 11.4 % of the marrow. These low values must be considered in the light of the fact that the phospholipid values are considerably higher



than those obtained in a more lipid selective reextract (cf. my own values) and thus perhaps too high owing to contamination with non-lipid P (see p. 29). Otherwise the experiences of RANDALL and the present author are largely in agreement.

The results on corpus callosum and intradural nerve roots reported early by KOCH and KOCH (106), obtained from a kind of dilute acid washed chloroform reextract and largely similar for man and dog, were respectively as follows (averages, on a dry weight basis): total lipids 64.3% and 64.5%; phospholipids (P determination) 28.8% and 36.04%; cholesterol (digitonin method) 10.25% and 11.52%. That these author's used a slightly higher conversion factor (25.77) for P in phospholipid does not explain their relative to my own much higher phospholipid values in nerve roots. The reason is obscure (cf. p. 110).

In adult human subjects SMITH and MAIR (163) found the following figures for grey and white matter, respectively (on a dry substance basis). total lipids 29% and 59%; phospholipids 15.3% and 22.7%; cholesterol 5.5% and 13.8%; cerebrosides 1.9% and 12.2%; »other lipids» 6.2% and 10.4%. As mentioned above (p. 31) the tissues were first formalin treated and then the lipids were extracted with chloroform. Phospholipids were determined by P analysis, cholesterol as digitonin precipitate according to WINDAUS. The cerebrosides were weighed after having been acetone extracted from a total lipid hydrolysate (by  $\text{Ba}(\text{OH})_2$  which would not appreciably change the cerebrosides). Judging by their figures, however, it seems probable that their cerebroside method in some of its stages causes losses, for the cerebroside values, especially in grey matter, are lower than other authors' and the unidentified lipids (obtained by difference) are correspondingly higher. The low phospholipid levels must be an effect of the formalin treatment. SMITH and MAIR already noted the absence of significant quantities of esterified cholesterol.

It would be valuable if by *quantitative isolation* it could be confirmed that the values obtained for various lipids by computations based on analyses of lipid constituents represent the lipid amounts actually present. In addition to the already mentioned experiments of SMITH and MAIR many more attempts have been made down the years with the aid of various fractionation methods. The most recent seems to be SCHWIRTH's (158). His method also embraces a careful analysis of the fractions isolated. Among other things he compares whole cerebrospinal marrow and brain of adult, showing that sphingomyelins are about 3 times as abundant in the dry substance of the former; a finding that only in part can be explained by the relatively higher contents of grey matter in brain. While this result is well compatible with the present author's

experiences, SCHUWIRTH's absolute level (2.7 %) for sphingomyelins in spinal cord is lower than that of the present author (by about 50 %). This also applies to the cerebroside level (5.7 %), while the cholesterol (17.3 %) and glycerophospholipid (27.0 %) levels deviate but little. The figures in brackets are means of two mutually fairly strongly diverging preparations, computed on a dry substance basis. Fractionation and isolation usually entail losses. The only question is whether SCHUWIRTH's procedure really can involve such a large and selective loss as 50 % of the sphingolipids found by the present author.

In his so called fat and cholesterol fraction SCHUWIRTH found glycerol values which transformed into molecular equivalents exceed the number of P equivalents present (the ratio is about  $\frac{2}{1}$ , cf. p. 47). However, the excess, calculated as neutral fat, is no larger than to correspond to less than 1 % of the dry substance. Using a less selective method, KAUCHER et al. (93) in whole ox brain found about 3 % neutral fat.

BACKLIN also employed fractionation (for methods see p. 87) and found in grey and white matter, respectively, from brain of adult rabbit the following values (calculated on a dry substance basis): total lipids 39.6 % and 52.3 %; phospholipids 29.1 % and 31.5 %; cholesterol 5.5 % and 10.6 %; cerebroside 5.0 % and 10.2 %. Here the surprising thing is the high contents of phospholipids in relation to the values of JOHNSON et al. and BRANTE. While in white matter it is fairly well compensated by lower contents of cholesterol and cerebroside, and consequently suggests defective fractionation, it is so high in grey matter that the total lipid value, based on the sum of analyzed lipids, exceeds the total lipid obtained by BRANTE by weighing. Therefore some methodological error must be suspected in BACKLIN's not particularly specific procedure, and this is especially pronounced as regards grey matter.

Results obtained by FRÄNKEL's fractionation methods (72) are difficult to compare with those given above. However, they too clearly indicate the great lipid preponderance in white matter as compared to grey, and to a certain extent the same thing in spinal cord white matter substance in relation to cerebral white matter (79 % total lipids in total spinal cord, 74.8 % in pure white cerebral matter).

As regards peripheral nerves other investigations are available in addition to the ones already mentioned in the above by JOHNSON et al. and SCHMIDT et al. In MAY's experiments with rabbit ischiadicus, which are described in detail in chapter IX, the normal values were as follows (computed on a dry substance basis); phospholipids =  $P \times 25$ ; phospholipids 15.2 %; cholesterol 7.8 %, or about 60-70 % of the present author's.

Is the difference due to variations in the removal of the connective tissue sheaths around the nerve — just as was assumed for JOHNSON's results —, the highly different extraction methods, or to differences in rabbit strains?

In human ischiadicus and spleen nerves of cow FALK (56) found respectively (% dry substance): total lipids 46.6 % and 11.5 %; lecithins 1.4 % and 1.1 %; cephalins 5.8 % and 2.7 %; cholesterol 11.6 % and 5.4 %; cerebroside 8.5 % and 0.7 %; other lipids 19.3 % and 1.6 %. While the values for total lipids compare well with the present author's most of the other figures, obtained by obsolete fractionation procedures, are highly different. FALK's results for spleen nerves are at least of historical interest in so far as they seem to be the only ones hitherto reported from this type of nerve, and have been frequently cited.

*Invertebrates.* Apparently the only more extensive chemical lipid analyses published hitherto are those of PATTERSON et al. (136) on honey-bee brain. In this material the optical properties of the nerve fibre sheaths are similar to those in crustacea (144). The average percentages on a dry weight basis were: total lipids 39, phospholipids (P-determination) 17, cholesterol (Liebermann-Burchard method) 1.3 cerebroside (orcinol method) 0, neutral fat about 2.5, unidentified lipids (possibly including some fatty acids) about 13. Lecithins were about 2 %, cephalins 12 % and sphingomyelins 1.5 %, but the authors point out that their figures for choline phospholipids probably are too low. The sphingomyelin values were obtained by the criticised reineckate method characteristics in the species.

Thus, as regards the preponderance among the lipids of phospholipids, the low cholesterol contents and the presence in negligible amounts if at all of cerebroside the results agree with mine from lobster. In respect of phospholipids, however, there are some differences: 1) the phospholipid percentage of total solids is about double, 2) lecithins amount to only some 12 % of total phospholipids while in lobster the corresponding figure is about 90 %.

The former difference may possibly be due to different contents of supportive tissue in the two nervous systems. The scarcity of lecithins in honey-bee brain may be an artefact arising from the too low choline values or from losses during the preparation (relatively prolonged storage in physiological saline). Naturally the differences may also be inherent characteristics in the species.

The absence of cerebroside in the cerebral ganglia of octopus has been established by LANFRANCHI (109), using Kimmelsliel's method (see p. 66).

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If the impressions from the literature are condensed and the explanations given of the divergencies in the results of other investigators remembered, it will be found that *the figures obtained by the present author probably well represent the true state of matters.*

## II. Writer's Results

### *Grey matter*

The values for central grey and peripheral (excluding lobster nerves) grey matter, recalculated on a total essential lipids basis, are in quite good agreement. Therefore, the great difference in the lipid contents of the two materials on a total solids basis mostly is due to a relatively higher contents of non-lipid solids in peripheral grey matter. Largely at least, this preponderance must be due to connective tissue which contains only little essential lipids in proportion to non-lipid solids (table XII) and is abundant in the peripheral nervous system (p. 105).

If it were possible to correct for the influence on the values of the connective tissue, the resultant lipid level, chiefly that of the axons, would probably not be very different for peripheral grey and central grey matter. In the lipid picture of the latter not only axons but also other processes and cell bodies of nerve cells and glia and blood vessels with contents contribute. With some right (191, 201) it may be assumed that the contents in the axons is produced continuously in the nervous cell and that the lipid contents in the latter is rather similar to that of the axons. Blood contains less than 1 % lipids and its negligible influence on the whole picture is therefore to lower the lipid values. As already mentioned only data of doubtful reliability could be obtained with respect to the amount of glia in the cortex but what information there is suggests that the quantity is fairly small (DONALDSON (44)). This being so, the cortex would give a lipid picture chiefly representative of the axon; this will be made use of in computations further below.

As regards lobster nerve its preparation at low temperature, preserving the native state, may have been of significance for its peculiar lipid analysis pattern.

### *White matter*

Here the decisive factors in the lipid picture are mainly the ratio axon volume/myelin sheath volume and the relative amount of supportive tissue. The latter factor is of minor importance in the central nervous system, but peripherally it may

be of great moment. The maximally high lipid values in the spinal cord may probably be dependent on a high sheath/axon ratio (N.B. averaged for *all* fibres) at the same time as the amount of supportive tissue is at a minimum. The reason for the higher lipid values in the spinal cord in comparison with brain white matter with its masses of short neurites with relatively thin or »no« sheaths is most likely a lower sheath/axon ratio, in comparison with peripheral nerves it is the considerable extra contribution of supportive tissue in these latter. The correctness of this interpretation is borne out by the relative increase in lipids typical of myelin sheaths (see below) in relation to lipids typical of axons from brain to the periphery of the spinal nervous system (which was pointed out above for sphingomyelin). These results thus indicate that the sheath/axon ratio may increase not only in the spinal cord as compared with cerebral marrow but also in spinal nerves as compared with spinal cord. But owing to a simultaneous increase of connective tissue between the fibres the latter increase does not yield a corresponding rise in the total lipid contents. I have not been able to find any representative histological data on sheath/axon ratios for brain and spinal cord white matter; own preliminary examination clearly shows pictures in the direction indicated by chemical means.

### III. Attempt to A Computation of Lipid Distribution in the Nerve Fibre

It should be noted that none of the lipids identified in marrow is wholly absent from vertebrate grey matter; the cerebroside contents of the latter is, however, with certainty slightly lower than the given figure which also includes gangliosides.

All the lipids are present in higher concentration in the wet marrow than in the wet cortex. If the axons are assumed to have the same composition in marrow and cortex, this should mean that all the lipids determined, to some extent at least, are contained in the myelin sheaths. If a lipid is predominantly located in the myelin sheaths, the ratio between its concentrations in marrow and cortex ought to be large, and vice versa. The table below contains the calculated means for cortex and white matter in man, cow, rabbit and rat, as well as the

multiples by which the various substances are more abundant in the marrow than in the cortex.

Table 6.

	Cortex		White matter		
	%	%	Brain	Spinal cord	
			Multiples of cortex value	%	Multiples of cortex value
Cholesterol	0.85	4.1	4.8	5.9	6.9
Cerebrosides	1.1	5.0	4.5	6.7	6.1
Lecithins	1.2	1.6	1.3	2.2	1.8
Cephalins A	1.9	4.5	2.4	6.1	3.2
» B	0.25	1.0	4.0	1.8	7.2
Sphingomyelins	0.35	1.2	3.4	1.9	5.4
	0.6		2.2		6.3
			3.7		3.7

It will be seen that cholesterol, cerebrosides and KOH non-decomposable phospholipids are about equally or 4.4 and 6.4 times as abundant in brain white and spinal cord white matter, respectively, as in grey matter. Cephalins A are only 2.4 and 3.2 times and lecithins not even twice as abundant. Apparently the best interpretation of this would be that the 3 groups mentioned first are essentially more characteristic of myelin sheaths = are »sheath typical». Not so with the latter groups which — and this applies particularly to the lecithins — in considerable proportions also are contained in axons.

Adult cortex always contains some myelin sheaths revealable by histological staining methods. Besides, in recent years it has been shown by means of polarization optical methods that most of the fibres found to be »unmyelinated» by staining methods in fact have thin equivalents of myelin sheaths. Young, histologically unmyelinated cortex contains about 88 % water, mature about 85 %. The average water contents of the myelin sheath has been calculated to about 50 % (44 a, 152). Then, if the reduction in the water contents in the cortex during maturation is caused by myelinization, the myelin quantity can be computed to 8 %. Similarly, calculated with the aid of its determined average water content, brain white will hold about 50 % each of axons and myelin sheaths, spinal cord white

about 34 % axons and 66 % sheaths. Assuming axons and sheaths to maintain their proper compositions in all parts of the nervous system, and the part of e. g. the cholesterol contained in the axon to be  $x$ , the part,  $y$ , which should be »sheath typical» can be roughly calculated as follows:

$$\begin{array}{lcl} \text{brain white: } 0.5x + 0.5y = 4.1 & \} & x = 0.23; y = 8.0 \\ \text{cortex: } 0.92x + 0.08y = 0.85 & \} & \\ \text{cord white: } 0.34x + 0.66y = 5.9 & \} & x = 0.16; y = 8.9 \end{array}$$

or in average  $x = 0.20; y = 8.4$ ;

Similarly computed, the axon and sheath contents, respectively, in % fresh tissue, will be: for cerebrosides 0.35 and 9.8, for lecithins 1.09 and 2.4 %, for cephalins A 1.37 and 8.1, for KOH non-decomposable phospholipids 0.24 and 4.8. But the cephalins are not a homogeneous group. By means of the averages in table 4, it is easy to calculate that the contents of ethanolamine cephalins in axon = 1.15 %, in sheath = 3.9 %. Then the rest of the cephalins, chiefly phosphatidyl serine, makes up 0.22 % in the axon and 4.2 % in the myelin sheath.

The ethanolamine analyses are few but the conclusions drawn from them are borne out by other results. The agreeing findings obtained by chromatography of the lipid fractions in grey and white matter have already been mentioned on p. 78. The separation into the different fractions must partly have been a consequence of differences in the degree of unsaturation of the fatty acids. This is known to be lower in phosphatidyl serine (IN. 33) than in phosphatidyl ethanolamine (IN. = 78 and probably still higher in some ethanolamine cephalins). The iodine number of phospholipid fatty acids in grey matter has been found by RANDALL (141) and YASUDA (199) to be much higher than in white matter. The figures of these investigators may be interpreted as pointing to a distribution of the serine and ethanolamine cephalins similar to that computed above from the present author's results.

According to the above, therefore, the so called sheath typical lipids would be cholesterol, cerebrosides, KOH non-decomposable and amino acid containing phospholipids and part of the lecithins and ethanolamine phospholipids. The small

amounts in the axon of sheath typical lipids may be an artefact. They disappear if the calculations are made on the assumption that myelin sheaths make up 10 % of mature cortex. This would be the result if in the above calculation of cortical myelin sheath content a small amount of thinly myelinated sheaths is considered to be present already in »young» cortex. As regards glycolipids the presence of a small amount in the axons seems more probable than it does for the other sheath typical lipids.

Of course, on account of the many assumptions necessary to make the computations possible, these may later show themselves to be faulty in certain details. For example, such high myelin sheath contents that have been assumed to be present in cortex is not easily compatible with histological experiences; other factors in addition to myelination may play a part in the dehydration mechanism during maturation of the cortex, etc. On the whole, however, the computed values are thought to represent the true state.

The picture of lipid distribution in axon and sheath which the author has obtained with the aid of the results in this chapter will be the following, the quantitative data presented only with considerable reservation.

The predominant axonic lipids are *lecithins* and *ethanolamine cephalins*. They are present in about equal amounts or approximately 1 % of fresh substance. Minute amounts of cholesterol and glycolipids may be present besides. The *myelin sheaths* contain a preponderance of *cholesterol* (25) and *cerebrosides* (29), but considerable quantities of *KOH non-decomposable phospholipids* (probably=sphingophospholipids) (14), *serine cephalins* (13) and *ethanolamine phospholipids* (12) as well as smaller amounts of *lecithins* (7) are also present. The average proportions in the myelin sheaths of brain and cord white matter are given in brackets in % of total essential lipids. Division by the molecular weights and  $\times 1000$  gives the following figures: 65, 35, 18, 17, 15 and 9; they are not far off the series 68, 34, 17, 17, 17 and 8.5 which should give the following molar proportions between the lipids in the average myelin sheath: *Cholesterol* / *cerebrosides* / *sphingophospholipids* / *phosphatidyl serine* / *ethanolamine ce-*



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*phalins / lecithins* = 8: 4: 2: 2: 2: 1. The sphingophospholipids are composed of about equal parts of choline containing and non-choline containing.

Lastly, the author would once more emphasize that these figures were worked out under the assumption that both the myelin sheath and the axon are essentially of the same composition in all kinds of nerve fibres. Even if this assumption holds for the most part, there are sure to exist discrepancies. I merely have to draw attention to the peculiar microscopical composition of the axon in spleen nerves and the olfactory bulb and to authenticated chemical differences between afferent and efferent, cholinergic and non-cholinergic fibres. Indeed, my own experiments have also shown up such differences; e. g. between evertbrate and vertebrate axons, between the ischiadicus myelin fibre and the average myelinated fibre as regards sphingomyelins, which are relatively more predominant in the former. Several authors have demonstrated a difference between the axoplasma in myelinated as opposed to unmyelinated fibres. Myeloaxoplasma has been thought to be an axoplasma, somehow modified by the simultaneous presence of the myelin sheath. Consequently, the author does not discredit the rather attractive hypothesis, that all the lecithins (and equivalent amounts of the ethanolamine cephalins) may be located in the axon but that they vary in concentration, being relatively abundant in strongly myelinated, long fibres, relatively rare in postganglionic, vegetative and short fibres. This would be an alternative explanation of the higher lecithin contents in cord white as compared to brain white matter and in white as compared to grey matter. It also would suggest an intimate relationship between myelin sheaths and the lipid contents of the axon.

The opposite possibility is that lecithins and phosphatidyl colamine, just as is believed of the other lipids, are wholly contained in the myelin sheath. In order to fit in with observed facts they must then be contained in a special sheath-like layer which during the myelinization increases but not at the same rate as the myelin sheath. During the myelination it could be built in in the myelin sheath or remain interior of the same. In the latter case it would be a matter of taste if it were regarded as a surface part of the axon or as an inner layer of the myelin sheath.

Mammalian axons, finally, could well be assumed to contain almost exclusively lecithins, just as is the case in lobster. But then the problem is where the abundantly present cephalins in peripheral non-myelinated mammalian nerve are located. It is difficult to imagine any other site than the axon. Can cephalins be formed postmortally from lecithins? Here it must be remembered that lobster nerves were throughout prepared in the frozen state, while at least 5 minutes passed after death before mammalian materials could be frozen. If during this short period half the lecithins can be transformed into phosphatidyl colamine, i. e. an extremely rapid demethylation, is difficult to say.

but seems incredible. Perhaps lobster axons are not at all comparable to axons of higher species.

### Summary

*Different portions of the central and peripheral nervous systems in several mammals and one evertibrate were analyzed for lipid contents. Particular pains were taken to obtain axon and myelin sheath materials as uncontaminated as possible. With the aid of the results an attempt was made to present a picture of the lipid distribution in axon and myelin sheath. Here, the chief lipids in the axon are lecithins and colamine-containing cephalins; in the myelin sheath cholesterol, cerebroside, sphingophospholipids and phosphatidyl serine. The myelin sheath also contains considerable amounts of ethanolamine cephalins and perhaps lecithins; the presence in the axon of some cholesterol and glycolipids is not absolutely out of the question.*

## Chapter VII

### Distribution of Lipids in Various Parts of the Nervous System during Development

In the first place these studies were intended to illuminate conditions in man. Suitable materials for the purpose could be obtained from operations and autopsies. Some corresponding materials were studied in rat which in respect of brain development (44 b) has been shown to reflect conditions in man very faithfully. In rat the effect of some avitaminotic conditions could also be studied and this was considered interesting as they could be assumed to induce changes in lipid development in the nervous system.

The following *development stages* were thought to be of special interest for the author's main problem: 1) the time for axon formation without simultaneous myelin sheath development, 2) the time for formation of myelin sheaths, 3) advanced age when some apparently physiological lipid changes are histologically observable.

1) begins in human brain during the 2nd. month of fetal life (86) and continues without significant admixture of myelin sheaths until about the 6th month; in rat the most lively formation of axons seems to take place during the first 10 days of life (43).

The development of myelin sheaths in the human brain starts regionally already during the 6th. fetal month but they are still sparse at birth; soon thereafter the myelination accelerates rapidly and from a histological point of view it is practically at a maximum within one year. In rat the histologically observable myelination starts in the spinal cord first, viz. on the 2nd. to 3rd. day of life; in rat brain it sets in only after 10 days. It then continues at a high and undiminished rate until about the 33 day and then successively decreases.

Incipiently already at 30-40 years of age, indeed even earlier in some parts, an increasing accumulation may be seen in man of lipid pigment in most nerve cells and later also glial cells. During the following two or three decades this pigment becomes so abundant that often the cortex assumes a dark brown colour; the change is at a maximum in senile dementia. The nature of the lipid is unknown; its normal source is a typical structure between the nucleus and the axon basis in the nerve cell.

The presence of choline or its metabolic prestages may be assumed essential for the formation of structures containing lecithin and sphingomyelin. Thiamine which plays a vital part in nerve metabolism, is localized to the myelin sheath (127) and deficiency of it has been reported to result in demyelination processes (169). The latter have also been seen to occur in pantothenic acid deficiency (169). Supposedly pantothenic acid is contained in choline acetylase (133 b). Inositol, of increased interest in connection with lipids since its recent discovery in animal lipids, e. g. in brain, and its demonstrated importance in the metabolism of liver lipids, has since long been suspected to be somehow involved in the formation of phospholipids. Already in connexion with some experiments with fungi the author had tested a structurally analogous inhibitor, gammexane, of inositol and in animals also it might antagonize the normal function of the metabolite in question. A-avitaminosis, finally, causes nervous degenerations which, however, are considered mechanic rather than metabolic in nature.

The author decided, parallelly with brain lipid development under normal diet conditions, to study same in rats obtaining a deficient supply of choline, thiamine, pantothenic acid and vitamin A, respectively, or injections of gammexane.

## Experimental

### Human material

*Fetuses* were obtained in immediate connexion with legal abortions. The brain was removed immediately, suitable parts were excised and frozen in »dry ice». The *outer layer* denotes a section about 1-2 mm thick of the external surface of the hemisphere wall, the *inner layer* the corresponding 2 mm thick layer of the ventricular surface, the *intermediary layer*, lastly, the centre part of the wall of the hemisphere after removal of 3-4 mm

on either side. The ages are approximate and are based on data from the patients and the lengths of the fetuses.

*Other human material* was derived from autopsies, the bodies having been stored for 1-3 days in a cold room after death. The cortical material was routinely taken from the medial side of the hemisphere, the marrow from centrum semiovale.

### *Human Subjects*

Age	Diagnosis and cause of death	Autopsy, days after death	Remarks
Premature 9 months	Bronchitis capillaris	2	Weight 2100 gm. Lived 6 days
Full term stillborn	Ruptura tentorii cerebelli	2	Weight 3300 gm.
2 months	Vitium org. cordis.	1	
3 "	Bronchitis capillaris	1	
4 "	Rhabdomyoma	—	
2 years	Oedema glottidis	2	
3 "	Coeliacia + Pedatrophia	1	Extremely emaciated. Hydropic degeneration in cortex cerebri
5 "	Barbituric acid intoxication	2	
10 "	Accident	—	
11 "	Ether narcosis	3	
12 "	Haematoma subduralae (accident)	1½	
16 "	Shot through heart	3	
19 "	Uraemia + Oedema pulmonis	½	
48 "	Final state after acute yellow liver atrophy	1	Some diffuse astrogliosis cerebri
55 "	Infarctus cordis	1	
55 "	Hypertonia	1	
65 "	Cancer vesicae + Embolia pulm.	3	
72 "	Haemorrhagia cerebri	—	
75 "	Nephritis chronica + uraemia	1	Cortex brown and atrophic
76 "	Infarctus cordis	1	
78 "	Anaemia	1	Cortex brown and atrophic
79 "	Cardiosclerosis	½	
90 "	Dementia senilis	3	

## Rat material

The animals were albino rats belonging to a strain since long in use at the Department of Histology, Upsala. They were reared in accordance with the usual instructions for dietic experiments (21). The development of the young was checked by weighing, etc. The basal diet was a food according to SURE (174, 175) containing:

<i>Casein</i> , vitamin-free and repeatedly boiled in alcohol-ether	750
<i>Cystine</i> , A. R.	15
<i>Butter</i>	350
<i>Glucose</i> (Dextropur)	1755
<i>Salt mixture</i> (176)	150

The components were thoroughly mixed in a rotary blender.

Daily adequate amounts in relation to age were given drop by drop with a pipette perorally of choline, thiamine, calcium pantothenate, niacin, riboflavine, biotin, and thrice weekly pure or synthetic preparations of vitamins A, D, E, and K.

The rats were grouped in litters. The control rats were put on a full diet as were their mothers during pregnancy and lactation. The experimental rats were given the same diet with the exception of the nutritional factor in question. Some choline deficiency rats were during the final weeks given glycocyamine in daily doses of 10 mM (37 days group) or 20-40 mM (45 days group and mother of 11 days group); in these groups 35 % lard instead of an equal amount of sugar was also employed. These latter measures were taken to intensify the deficiency in labile methyl groups (170). Gammexane, in the form of a crude product (10 %  $\gamma$ -isomer) dispersed in propylene glycol, was administered in daily doses of 0.3-2 mg by means of subcutaneous injections into the mother during pregnancy and the young after birth.

The groups: control 70 days, thiamine deficiency 70 days, A-avitaminosis 70 days were obtained from the Swedish State Institute of Public Health and belonged to the WISTAR strain. They had been given an otherwise adequate diet, with or without the addition of vitamins A and B<sub>1</sub>.

The control rats apparently developed fully normally. In the rats on deficient diets the following **deficiency symptoms** were observed.

### *Choline deficiency rats*

**11 days.** Average body weight 6.2 g. Consequently the young had not put on more than about 1 g after birth (weight of control rats: 1 day 5.2 g, 11 days 19.2 g). When killed they were moribund and so feeble as to be almost incapable of movement. The stomach of solitary animals contained milk, but judging by the appearance of the intestines the intake of food probably had been substandard. The internal organs exhibited no special signs of choline deficiency (no hemorrhages, no fatty infiltrations). The mother had a heavy fatty liver.

**17 days.** Normal development. No symptoms of choline deficiency.

**24 days.** Normal development. No symptoms of choline deficiency.

37 days. Were killed, moribund, after about 1 week of intensified methyl deficiency diet (see above) during which they rapidly lost weight and towards the end developed severe tremor (uremic?). Massive renal hemorrhages in all the animals, slight or no fatty infiltration in the liver. The nervous system was macroscopically normal.

45 days. Some reduction in weight towards the end, otherwise normal development. No symptoms. Slight fatty infiltration in livers, other organs normal.

#### *Thiamine deficiency rats*

17 days. Possibly slight lag in weight, otherwise no symptoms. Internal organs normal.

24 days. As for 17 days group.

40 days. Lost weight steadily during final 19 days. Towards the end classical symptoms of beri-beri with spasmodic rigidity of the hind legs and spastic-ataxic locomotion. Moribund when killed.

70 days. Considerable weight reduction. Otherwise no obvious B<sub>1</sub> deficiency symptoms.

#### *Pantothenic acid deficiency rats*

Apart from a considerable weight lag in both groups and a tendency to rhinitis and diarrhoea no changes. No nervous symptoms.

#### *Gammeexane rats*

Despite increased doses of gameexane the development of these animals was fully normal. No nervous symptoms. Enlarged liver was a general postmortem finding. In some mottled liver, otherwise nothing remarkable.

#### *Vitamin A deficiency rats*

Loss of weight and bad general condition at time of death. No nervous symptoms.

#### *Preparing the material to be analyzed*

3-12 rats per group were used for each analysis. The rats were killed by decapitation. The skull was immediately opened and the brain cut off from the spinal cord at a place caudally in the medulla oblongata and transferred whole to glass vessels in 'dry ice'. This procedure took about 5 minutes. Then the spinal column was cut open and the spinal cord separated from other tissues and removed in entirety. Membranes and nerve roots were removed and the spinal marrow also was chilled in 'dry ice'. The whole programme for each animal took about 10-15 minutes.

## Results

These appear in tables IX and X.

### I. Human subjects

#### *Fetal stages.*

The difference between the part which later becomes cortex (*outer layer*) and that which turns into marrow (*inner layer*) is but little pronounced at this stage of development. There is a difference, however, and that as early as in the 4th. month of fetal life. While the cerebroside results are open to some questioning, the absolute phospholipid and cholesterol values are higher in inner layer; this is especially evident when calculating on a total non lipid solids basis; the proportions between the various lipids are more equivalent for the two layers. A 3rd month fetal brain was examined whole and the values did not deviate significantly from those in the four month stage. In later stages the difference between the inner and outer layer grows steadily; the contents of lipids in solids in the latter increasing but little before birth while that of the inner layer increases markedly. The *intermediate layer*, which was studied in a 7th month fetus only, exhibited a lipid picture most nearly corresponding to that of the inner layer.

During fetal development the lipids, and this applies to all the layers, are mostly composed of phospholipids, while the cholesterol contents merely are  $\frac{1}{4}$ - $\frac{1}{5}$  as high and the cerebro-sides, although present, lie on a still lower level. The phospholipid group throughout consists of about equal parts of substances with or without choline; the KOH decomposable ones are strongly predominating. A small quantity of sphingomyelin seems to be consistantly present already from the 4 month stage. However, mostly it is so minute that it might be at least largely an artefact due to differencies in the determination of lecithin choline in relation to total choline, discussed on p. 61. The same thing could be suspected to apply to the whole group of KOH non-decomposable phospholipids whose level judging by findings discussed on p. 98 probably to some extent depends on the circumstances obtaining during the preparation. This view is contradicted, however, by the value for glycerol as phospholipids which by difference suggests the presence of a small



quantity of non-glycerophospholipids. »Diglycerides» are at a minimum or absent before birth in both outer and inner layer.

Thus, in human brain, from the 3 month stage until birth, the »essential lipids» amount to 20-25 % of total solids; in the inner layer they increase successively in relation to other solids while the increase is small in the outer layer during fetal development. The »essential lipids» are composed of 70-75 % phospholipids; 20-25 % cholesterol and about 10 % cerebrosides. The phospholipids are made up of about equal quantities of lecithins and cephalins while the sphingomyelins are almost negligible. Evidently there are no neutral fats nor esterified cholesterol. The »unidentified fraction» is quite considerable.

### *The neonatal period*

*Cortex.* While during fetal development the lipid contents in the outer layer as mentioned only changed insignificantly, it increases rapidly after birth and attains values in most respects comparable to those in normal adult cortex already during the 3rd. month. During this process KOH non-decomposable phospholipids and sphingomyelins increase so that the presence of these groups no longer can be doubted. Possibly cephalins increase slightly at the expense of lecithins; some »diglycerides» appear during the 2nd. and 3rd. months.

*Marrow.* In premature the marrow lipid content was unexpectedly high (which possibly may be due to faulty dry substance determination). Otherwise a continuous, rapid development is the rule. This is especially true of cholesterol and cerebrosides which more than double in quantity during the first 3-4 months. At the same time the phospholipid level increases by less than 50 %. This increase almost wholly centres on the cephalins, more exactly the KOH non-decomposable ones. KOH decomposable phospholipids seem to have attained their final level already at birth. »Diglycerides» occur fairly abundantly in the 2-4 months stage but they do not parallel the increase in cephalins B very plainly. The high »unidentified fraction» at 3 months should be noted since connected to the »diglyceride» value it bears out the *possible presence of neutral fat*. Cholesteryl esters were present in small amounts during the first months; later they were absent. It was tried to

excise the purest white matter possible from brain of 2-4 months but owing to the thinness of the layer it was difficult to avoid some admixture of non-myelinized future marrow tissue. The fact that under such circumstances values are obtained where the lipid proportions so closely agree with those in adult white matter suggests that *myelin already at the moment for its laying down in the sheaths has much its definite composition* (perhaps the phospholipid constituents are an exception, cephalins B being unusually abundant, sphingomyelins sparse).

Thus, it is plain that the definite proportion of the various lipids in total solids is largely arrived at during the neonatal period both in pure white substance and cortex. Owing to the still high water contents the values on a fresh substance basis are as yet lower than the definite ones.

#### *Childhood and youth*

*Cortex.* Apart from a reduction of the water contents no clear lipid changes take place here as compared to earlier stages.

*Marrow.* The proportions of cholesterol, cerebroside and sphingomyelins increase, lecithins decrease in relation to earlier stages. *The brain of a 10 year old exhibits much the adult composition* in all the respects analyzed, excepting possibly cerebroside which seem to increase slightly even in the following.

#### *Advanced age*

*Cortex.* The somewhat varying values on the whole lie on a slightly higher level than in younger stages, but in most cases the difference is negligible (except for the 72 year old with the phospholipid increase) and it cannot be said that there is a special tendency to increase of any individual lipid.

*Marrow.* Except for a slight cerebroside increase no differences could be observed in comparison with the values in youth. The case with senile dementia had a higher »unidentified fraction» than usual, in marrow as well as in cortex.

## II. Rats

### A. Controls

*Brain at birth* contains a little cholesterol and cerebroside but much more phospholipids. These latter are almost exclusively KOH decomposable and consist of approximately equal amounts of lecithins and cephalins. Cholesterol amounts to

about 16 % of total essential lipids, cerebrosides to 11 %. At 11 days these proportions remain unchanged; in the phospholipid group, on the other hand a considerable proportion of KOH non-decomposable phospholipids has developed. All the lipids have increased considerably relative to other solids. The picture at 17 days scarcely at all differs from that at 11 days. Neutral fat seems to be practically absent in the early stages; a little cholesteryl esters may be present.

*In spinal cord* things are much the same during the first 17 days. Thus, at 11 days all lipids have increased, cholesterol a little more than total phospholipids, cephalins more than lecithins. The biggest increase, however, its noticed in KOH non-decomposable phospholipids. This fraction is also responsible for most of the increase during the *next 7 days* in which period the amounts of other lipids remain practically constant. During 17-24 days cholesterol and cerebrosides increase overwhelmingly; during the *remainder of the observation period* there is seen a decelerating increase of all lipids except lecithins.

*The cortex* was studied separately only from a relatively late stage, i. e. at 24 days, and then it had almost attained its adult composition as far as lipids go. Henceforth there was only a slight increase comprising all the lipids but most pronounced for cholesterol, cerebrosides and sphingomyelins.

### B. Diet deficient rats

As may be observed on p. 127 the deficiency disorders obtained in the various groups were different in degree. The animals subjected to gammexane administration would scarcely have been damaged since the dosages was on the small side and the symptoms conspicuous by their absence. Those of other groups, *where manifest symptoms of deficiency were seen*, were: choline deficiency, pronounced in 37 days group, insignificant in 45 days group; thiamine deficiency, pronounced (with nervous symptoms) in 41 days group, moderate 70 days group, vitamin A deficiency of moderate intensity. No definite pantothenic acid deficiency picture could be seen. The state of deficiency in the choline 11 days group may have been one of undernutrition. (Was lactation depreciated by fatty liver in mother?) *The remaining animals could be considered almost unaffected.*

Apart from small deviations the lipid values for the rats described in these experiments run largely parallel with the corresponding values for control rats. Thus, for example, *no change could be seen in the very ill beri-beri 41 days rats or in the choline deficiency 37 days rats with renal hemorrhages*, nor was there any plain change (on a total solids basis) in 11 days choline deficiency rats in which the body weight had remained practically the same since birth (their nervous system, however, weighed much less than that of the 11 days controls and therefore its absolute amounts of all nerve lipids were considerably less than those in the controls). It was less surprising that the A-avitaminosis, the gammexane, and the pantothenic acid deficiency did not give rise to any changes. In the author's opinion the entire table contains *only two groups in which plain deviations from the normal appear*. They both are to be found among the thiamine deficiency groups. One of them consists of a much reduced cerebroside value in 70 days spinal cord; the relatively low total lipid value likewise points to some decrease in lipids. However, the brain stem in the same animals upon analysis turned out not to have a lower cerebroside value than brain stem from controls. The absence of cerebroside changes in other thiamine deficiency groups also bespeaks the accidental nature of the cerebroside reduction in the 70 days group. In the 17 days thiamine deficiency group the spinal cord exhibits a phospholipid picture which approximately corresponds to that occurring normally shortly after birth, i. e. the phospholipids, especially those KOH non-decomposable, are lower than normal for the age. The »unidentified fraction» on the other hand is increased and the impression therefore is obtained that lipid prestages of the phospholipids had been synthesized but could not be further developed. The conditions in spinal cord, however, are by no means paralleled by any similar changes in brain, and one solitary finding does not permit any conclusions to be drawn.

*The results of the dietary experiments may perhaps best be looked upon as a new proof of the priority of the nervous system at the expense of other tissues as regards the utilization of nutritional factors.* Consequently, the results can therefore to a large extent bear out and support the correctness of the observations made in the control animals.

## Discussion

### A. On the Normal Development of Lipids in Nerve Tissues

#### I. Earlier Experiences

##### a. On human subjects

Availing themselves of KÖCH's procedure, MACARTUR and DOISY (115) carried out extensive studies on various brain parts during several developmental stages. Those of their oft quoted results of particular interest here refer to fetal stages when, as is known, no considerable differentiation yet has taken place in marrow and cortex. In 3 months fetus, 7 months do. and 1 month child they report the following respective values (in % of total solids): Total lipids 22.1, 26.3 and 31.2; phospholipids 12.9, 13.1 and 16.3; cerebrosides 0, but sulphatides (reckoned to contain 40% cerebrosides) were 2.0, 2.9 and 2.1. Later all values increased steadily. Considering the circumstances the agreement with the present author's values must be regarded as rather good.

SCHUWIRTH (156), using the fractionation procedure mentioned on p. 114, gives some interesting data on human 7-8 months fetus and newborns. In % of dry substance he finds total lipids to be respectively 23.9 and 20.3; phospholipids 16.9 and 15.8; cholesterol 6.8 and 4.4; cerebrosides 0.02 and 0.05; gangliosides 0.1 and 0.4. The phospholipids were all glycerol containing ones; consequently there were no sphingomyelins. *In the fetal brains*, on the other hand, he found *not inconsiderable quantities of lignerocylsphingosine*, which was totally absent in later stages of development. The »fat and cholesterol» fraction contained glycerol and P in the approximate molar proportion 3:2 (c. f. p. 47). If 1 molecule of glycerol is assumed to be bound per atom of P the contents of neutral fat would then be less than 0.8 % in both materials. Just as was the case with spinal cord (see p. 115) SCHUWIRTH's values for sphingolipids, therefore, owing to their minuteness, deviate strongly from the present investigator's. The cholesterol values deviate in the other direction. Other findings are in agreement, e. g. that practically all the phospholipids contain glycerol and that neutral fats are absent or low. The cerebroside value is in BRANTE's experiments calculated on the basis of reducing substance to which gangliosides also contribute. Moreover, the latter contain twice as high percentage of hexose as do cere-

brosides. Employing a method different from SCHUWIRTH'S, viz. KLENK'S neuraminic acid method, the author in collaboration with SVENNERHOLM (28) has found in 7 and 8 months fetus 0.4 and 0.5 % gangliosides, respectively. Converted into cerebroside this would correspond to about 0.8 and 1.0 %. At this stage, therefore, a great part of the present author's cerebroside values, and consequently of the difference between BRANTE'S and SCHUWIRTH'S cerebroside results, may consist of gangliosides. (Being relatively water soluble these should also affect the differences in cerebroside values obtained by BRANTE'S as opposed to KOCH'S and SMITH and MAIR'S methods:)

LORRAINE-SMITH and MAIR (163) seem to be the only investigators up to the present who have studied human white and grey matter separately during development; however, only from birth and onwards. Their results during early stages of development have been collected in table 7 and will be dealt with slightly before passing on.

Table 7. *Lipids in Dry Substance at Different Ages according to SMITH and MAIR (163). (in %)*

	Full term stillborn		3 months old child		14 months old child		5 years old child		Adult human	
	Grey	White	Grey	White	Grey	White	Grey	White	Grey	White
Total lipids	25	31	31	40	29	53	31	51	29	59
Phospholipids	7.3	16.5	15.1	19.5	15.9	22.3	17.5	20.9	15.3	22.7
Cholesterol	4.5	7.0	5.1	7.3	6.2	13.0	5.0	12.5	5.5	13.8
Cerebroside	0.7	1.2	0.7	3.6	1.3	9.9	1.3	8.3	1.9	12.2
Unidentified	12.5	6.4	10.1	9.6	5.7	8.4	7.2	9.3	6.2	10.4

It must be remembered that the brains were formalin preserved, which must have caused losses in phospholipids. True enough, as compared with the present author's, the phospholipid values are low all along the line; »unidentified fraction» correspondingly higher in more or less the same degree. The relatively higher cholesterol values in the early stages can also very well be attributed to the formalin preservation treatment; not so with the low cerebroside level. The latter should have some methodological background (cf. above and p. 136).

#### *b. On rat*

The classical works in this field are those of KOCH (104) and KOCH and KOCH (105). In the fetal brain of pig they found about the

same composition at 40 as at 60 days (probably corresponding to about 3 and 5 months for human fetus), including about 15 % phospholipids in total solids. At 90 days the phospholipid value had increased to 17 %.

Owing to many circumstances newborn albino rat was considered to correspond to 60 days pig fetus as regards degree of development. The chemical composition of the brain also agreed, among other things as regards phospholipids. No cerebrosides were to be found. During the first 10 days after birth, when axon formation and cell growth are rapid, there was considerable phospholipids built up and still higher protein formation in rat brain, thereby rather decreasing phospholipids in % of total solids. During the next 10 days, when the myelinization is most intense, the phospholipids increased very rapidly and the percentage in total solids went up to about 21. Thereafter the rate of myelination decreased progressively and no noteworthy increase of phospholipids set in. The cerebrosides, on the contrary, increased from about 3 % at 20 days to 6 % at 40 and 8 % after 120 days, all on a total solids basis.

Thus, the present author's results on whole rat brain deviate from KOCH's by a) the presence of glycolipids already at birth, b) a considerable phospholipid increase in lipids on total solids already during the first 11 days whereafter the increase slackens off. It is improbable that the reason for the latter divergency is due to the 1 day longer period of development. At birth KOCH found 1.5 % sulphatides, 40 % of which, judging by the method of determination, must be cerebrosides. These, therefore, in part correspond to the cerebroside values in my analyses in which no subdivision into S-containing and S-free cerebrosides was made. Has the rest of the cerebrosides (or possibly the gangliosides) been lost in some phase of the procedure (the water washings at lipid precipitation?, the hydrolysis)?

WILLIAMS et al. (194) have recently studied lipids in rat tissues and among them brain during development from 15-70 days. In % of dry weight their 15 days values were as follows: total lipids 32.6 %, phospholipids 21.3, cholesterol, free 4.4, total 4.7, cerebrosides 3.8, choline phospholipids 11.0, cephalins 10.4, neutral fats 2.80; accordingly the agreement is very good with the present author's results for the 11-18 days period. with the exception of the somewhat higher choline phospholipid and neutral fat (same unspecific method as KAUCHER et al., see p. 115) values of WILLIAMS et al. As their sphingomyelin values were obtained by a method recently demonstrated to be highly unsatisfactory (79), they will not be discussed. Contrary to KOCH they find the phospholipids in total solids considerably increased from the 15th. to the 45th. day and

then still more up to the 70th. day (27.2 %). The increase solely consisted of cephalins while choline phospholipids decreased slightly. Proportionally, cholesterol and cerebrosides increased most (70 days values 7.1 and 8.4 %; only free cholesterol present). The present author possesses analyses only of cortex and spinal cord during the latter two advanced stages. Judging by them, it is obvious that the increased lipid concentrations after 15 days may not be attributed to cortical development, nor was this to be expected. WILLIAMS et al.'s results, recalculated by the present author, have been used for completion of the diagram on p. 140-41.

As regards the phospholipid group my spinal cord values are on the whole well agreed in all the three stages; my cholesterol and cerebroside values increase quicker and more; an observation that should be a function of the greater proportion of myelinated tissue in the spinal cord.

WAELSCH et al. (186) have determined fatty acids and unsaponifiable lipids during early rat life, finding, in agreement with my own figures on cholesterol, that already during the first 12 days the unsaponifiable fraction increased continuously in relation to other solids. Probably their consistently slightly lower values can be ascribed to methodological reasons. Fatty acids in % of solids, on the contrary, remained unchanged during the same period. Assuming the fatty acid and phospholipid molecular weights to be 302 and 775, respectively, they obtained an average phospholipid value (calculated from fatty acids) during the first 12 days = 13.3 % of dry tissue, i. e. not far from my own value at birth. But my phospholipid values had increased markedly already after the first 11 days (also true of the choline deficiency group). Possibly this deviation from the finding of WAELSCH et al. may be accidental, perhaps due to dissimilar development in different rat strains or individual litters, and this suggestion is supported by the above described experiences of KOCH. Morphologically, the period is characterized by general growth of the neurocellular cytoplasm and no myelination as yet, i. e. there are no histologically observable reasons for an altered chemical composition if the axon is assumed not to be differently composed than the rest of the neuron. This problem is further discussed below (p. 146).



## II. Writer's Results

### *Comparison between man and rat as regards lipid development in nervous tissue.*

Direct comparisons can be made only as regards whole brain in three months human fetus and 1 day old rat brain and as regards cortex. Other tissue samples are not equivalent. From the point of view of morphological development 1 rat day may be considered equivalent to 30 human days. A newborn human subject is about as old as a 5 days old rat (DONALDSON and coll.). The 3 months human fetus therefore corresponds to a rat 1  $\frac{1}{3}$ rd. days before birth. A 1 day old rat brain, therefore, should contain slightly more than the human fetal brain of directly comparable lipids, and such was actually found to be the case. The cortex in 24, 41 and 70 days old rat should be compared with that in 1  $\frac{1}{2}$ , 3 and 5 years old child; on the whole the correlation is satisfactory. Probably this applies generally to lipid development in the nervous system (cf. MACARTHUR and DOISY 115)).

This assumption is borne out by a special similarity as regards the development of some lipids in human white matter and rat spinal cord which, to be sure, mainly is composed of white matter. *During the more active phase of the myelinization process both contain fairly pronounced accumulations of cephalins B*, which then alone are responsible for much the largest part of the KOH non-decomposable phospholipids. Later the sphingomyelins increase although the entire KOH non-decomposable group remains fairly constant, cephalins B therefore being reduced gradually to adult values. Hence it is easy to regard cephalins B as being, at least in part, choline-free prestages of sphingomyelins. A »diglyceride» increase, which parallels the rise in cephalins B, suggests that at this stage cephalins B may contain some KOH non-decomposable glycerophospholipids, while a simultaneous but inconstant increase of the unidentified fraction points to the presence of neutral fat or other non-analyzed lipids (cf. SCHUWIRTH's finding of ceramides, p. 134).

My investigations on rats complete and extend the scope of KOCH's. The results were recomputed with the aid of observed weights or weights taken from DONALDSON's book »The Rat»

(43) so as to apply to whole brain and cord, respectively, and are illustrated in diagrams 13 and 4. It may be assumed that the curves for corresponding human tissues would have a similar course.

### *Cortex development*

Human cortex contains no histologically observable myelin sheaths until several months after birth. Such being the case it is interesting to note that from the 4 months fetal stage to 2 months infancy the lipid proportion noticeably increases and relatively more for cholesterol and cerebrosides than for phospholipids. In the author's opinion this may possibly be a consequence of the axon formation and infiltration taking place in this very period; the mechanism will be discussed later.

Naturally, the lipid increase may also be interpreted as due to a relatively low protein level in axons or as some kind of unexplained »maturing» process for the cellular contents of the cortex. Already during the neonatal period the latter, owing to the early lipid increase, attains a composition which subsequently changes but little. This is surprising with respect to advanced age because then, as mentioned previously, marked lipid accumulations can be detected by histological means. The nerve cells are often distended like balloons by sudan staining lipids. Since these cells constitute such an insignificant fraction (p. 104), of the entire cortex volume, however, the lipid changes in them may not be detectable by methods like the ones adopted by the present author. An alternative interpretation of the results is that the normal lipids for some reason have become increasingly visible, e. g. by being released from lipoprotein compounds and, why not, also owing to inadequate transfer to the axons, possibly their normal route of removal (191, 192, 201)!

### *Marrow development*

The formation of brain marrow is commenced by infiltration of axons from the cortex into the intermediate layer, in which in 4 months fetus the internal, so called striated layers already have developed; later these layers are the first to become myelinated. They are located laterally of the ependymal layer

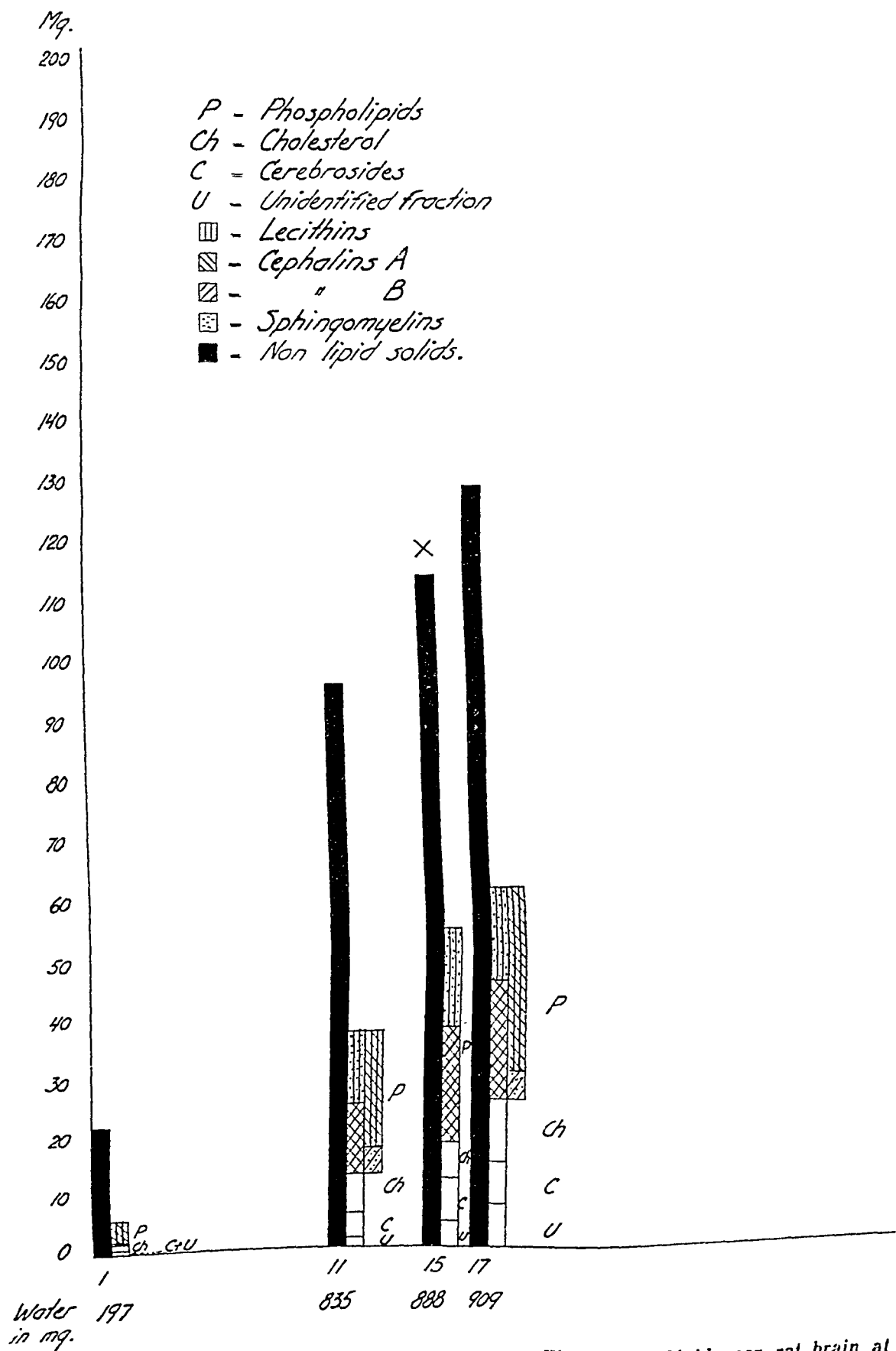
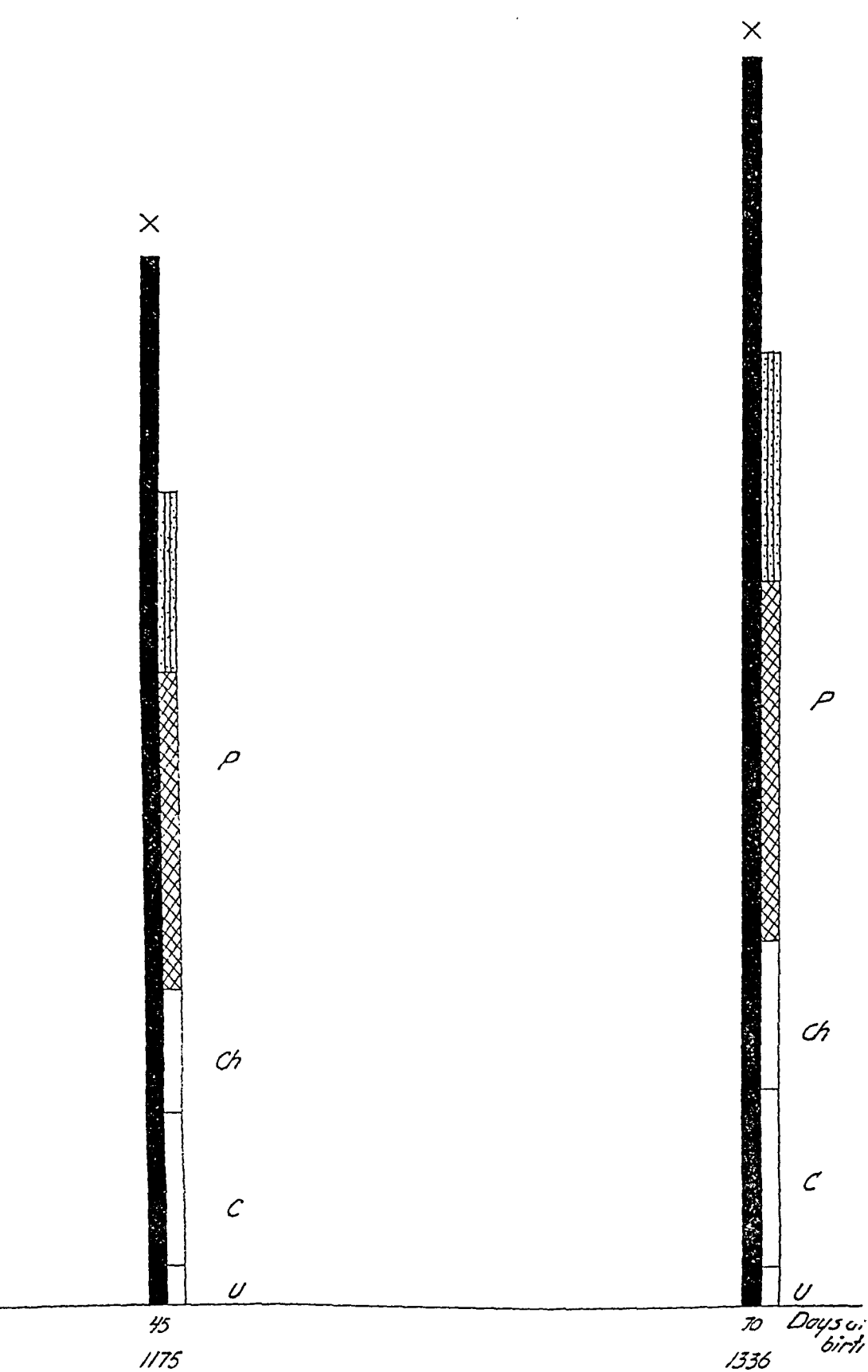


Figure 3. Lipids per rat brain at

× = calculated from the result of WILLIAMS et al. (194).



ferent stages of early development.

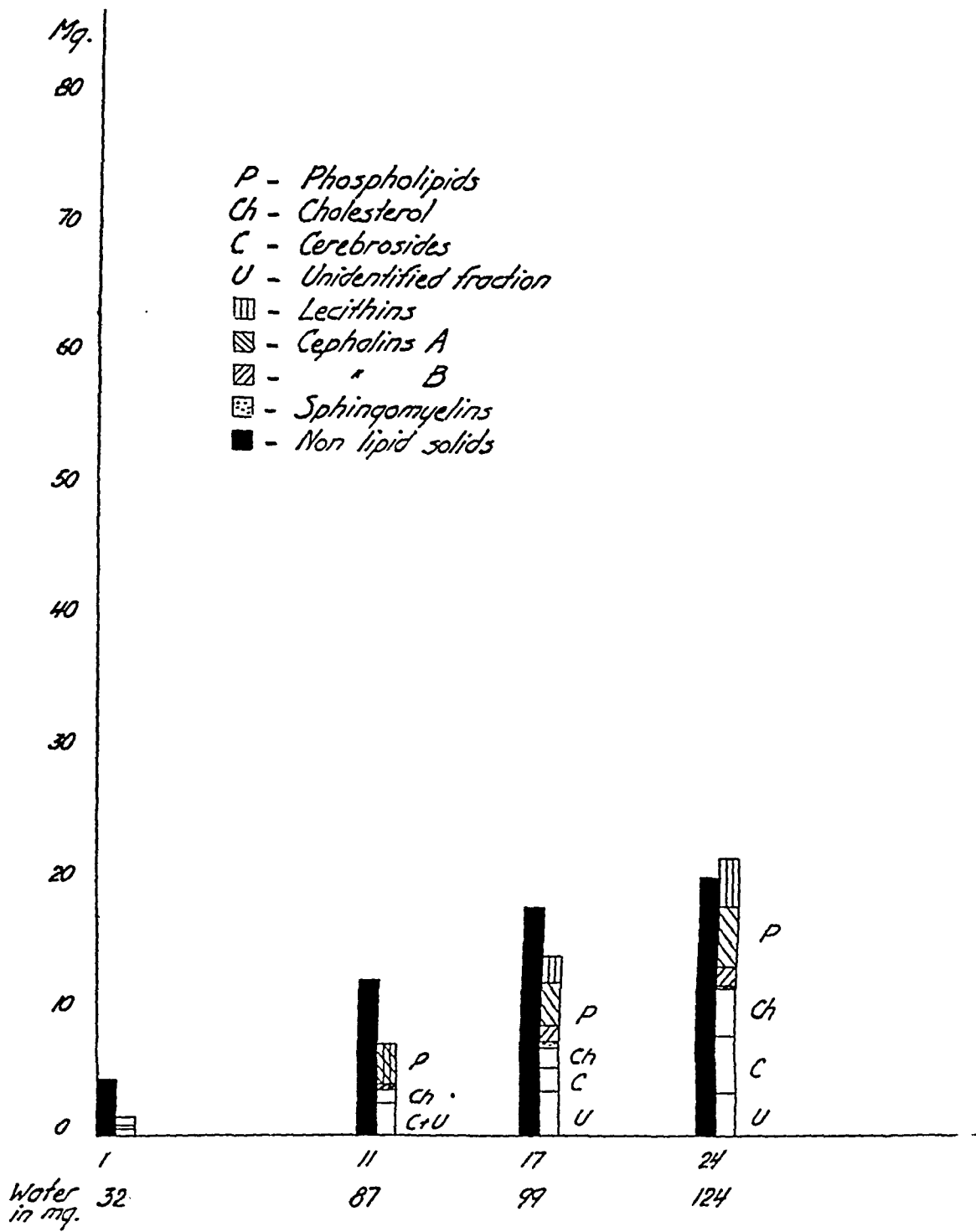
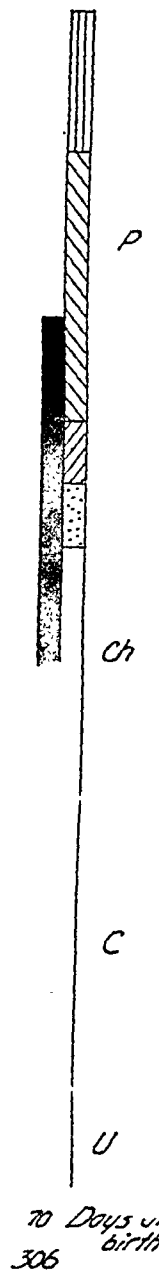
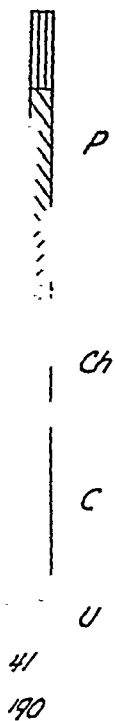


Figure 4. Lipids per rat spinal cord at



*different stages of early development.*

and are in this early stage about 1.6 mm thick, whereas the ependymal layer is only 0.2 mm thick. Thus, my so called »inner layer» includes both the future white matter and a small quantity of ependyma. It is not surprising, therefore, that the intermediate layer (the infiltration zone of the axons) analyzed like the inner layer. From the 4th. until the 6th. fetal month the infiltration of axons into the inner layer continues, and the increase in lipids during the same period is probably due to these axons. Thereby, remarkably enough, the marrow already at this early stage attains such high a lipid level as the cortex assumes only after birth and, strange to say, without histologically detectable myelination. The assumption that could be made already in the discussion on the cortex that the axons are richer in lipids than their parent cells (which practically alone make up the cortex at this early stage) is further supported hereby. In fact, also in the earliest stages studied the future marrow has consistently a higher lipid concentration than the corresponding cortex layer (p. 129).

After the 6th. fetal month lipid development progresses continuously, and now it is partly caused by commencing, histologically observable, myelination. This gathers speed only after birth, however, which appears to full evidence in the tables.

During myelination and especially at its maximum, in the 3rd.-4th. month, an accumulation of strongly sudan staining lipids appears in the marrow, in man regularly, in certain animals also but less marked histologically. In some camps this lipid deposition has been considered pathological and due to parturition trauma (VIRCHOW's *congenital neonatal encephalitis*), but the more recent view is that it is a phase in the normal myelination of the nerve fibres (147, 183). This is indicated by, e. g., the facts that at least partly the »fat» is arranged in narrow strips along the axons, and that otherwise the tissue appears normal. According to RYDBERG (147) the »fat» is greatly pericellularly localized, predominantly around glia cells and in the neighbourhood of vessels, and in his opinion it is composed of myelin prestages, deposited from the tissue fluid upon the surface of glia cells or nerve fibres.

If this sudan staining premyelin were inherently different from final myelin, the results of my lipid analyses in cor-

responding stages would show some typical upward alterations from normal. As mentioned previously, in man as well as in rat, the following were noticed as changes peculiar for the myelination period: a considerable rise in cephalins B (sphingophospholipids before choline esterification?), a slight »diglyceride» (neutral fat? diglycerophospholipids?) increase and a variable upward tendency of the unidentified fraction (ceramides? glycerides?). These groups (about other possible contents see p. 21) may, therefore, have something to deal with the visible changes and thus with »premyelin». SCHUWIRTH's finding (see p. 134) of ceramides in human fetuses should be remembered in this connection.

*Comparison between brain and spinal cord as regards lipid development*

The growing importance of the lipids in comparison to other solids as the age increases is illustrated by the diagrams in figs. 3 and 4. This is particularly noticeable in the spinal cord, the non-lipid solids in which are predominant during the first 20 days of life after which the leading position as regards concentration is gradually taken by the lipids. Undoubtedly, this phenomenon is due to the increasing myelination and ingrowing of centrally and peripherally arising myelin fibres. Nor can it be doubted that the deviating course for chemical development in cord as opposed to brain chiefly is due to the increasingly greater relative proportion of white matter and thus of myelin sheaths in the cord than in the brain. If with DONALDSON (44 a) the water contents of myelin sheaths is assumed to be 50 % and of unmyelinated grey matter 88 %, then with the aid of DONALDSON's figures for water contents (43) it will be found that whole brain after 1 day contains practically no sheaths, after 11 days about 5 %, after 17 days 14 %, after 24 days 17 %, after 41 days 23 %, after 70 days 24 %, after 1 year 28 %. The corresponding percentages for spinal cord are 4, 13, 19, 26, 37, 43 and 50, respectively.

11 days brain contains a small amount of histologically observable myelin sheaths (apparently only in brain stem (43) and certainly amounting to less than 5 % of the total brain mass), otherwise it is histologically different from 1 day brain chiefly in its *very great increase of nerve cell branches*, especi-



ally axons, in relation to the cell bodies proper. The simultaneous increase in lipids in proportion to other solids (which are unchanged on a fresh substance basis) is too great to be explained even by the formation of 5 % myelin sheaths.

#### B. Considerations as to the Lipid Contents in the Developing Axon

In 3 different materials (see p. 139, 144 and above) it was observed that *an increase of the axon contents without simultaneous visible myelination in comparison to the cell body contents in a tissue results in a raised lipid proportion of solids* in the tissue. This was usually not due to a decrease in non-lipid solids of fresh tissue. Consequently lipids actually seem to be more abundant in axons than in axonic parent cells. The reason may be, that the cytoplasm contains more lipids than the nucleus, or the increased surface area if it is assumed that the lipids are more concentrated there. The increase is relatively greater for cholesterol than for phospholipids, perhaps suggesting that the former is more abundant than the latter in the surface and/or throughout the cytoplasm; the latter alternative seems less probable in the light of the findings in chapter VI. If data were available for the surface it would by means of the analysis results be possible to calculate the proportion of lipids possible to be contained in a surface layer; it may be surmised, however, that the increase in surface area due to branching of the cortical cells and ingrowing in the cortex of axons is too great to permit a particularly large portion of the lipids to be so localized.

Howsoever the lipids are in detail distributed, it is fully clear from the investigations in this chapter that *the axons on the whole contain a similar combination and quantity (by no means less) of lipids as their parent cells*. This is true of fetal life and nothing suggests that such would not be the case later also.

On a fresh substance basis the axon developing in the fetus contains about 1.5-1.6 % phospholipids, about 0.4 % cholesterol and 0.2-0.3 % glycolipids. Here almost 90 % of the phospholipids are KOH decomposable and the lecithins are predominant, amounting to 0.7 % of total phospholipids. These values

are still far off those attributed to the axon in mature cortex. Supposing that, as was done in the case of the latter, p. 119, some of the lecithins are localized in a sheath equivalent, that this contains also all the cholesterol and, finally, that the ratios of cholesterol to lecithin and to cephalin are the same as those calculated above for mature myelin sheath then a simple numerical operation yields the result that during fetal life the lecithins amount to 0.6 % of the immature axon, 0.1 % of the »sheath». The corresponding figures for cephalins are 0.3 and 0.4 %. Thus, the values would be much lower than those calculated for mature cortex. The calculations as to fetal tissue may, however, be wholly misleading due to special conditions during development. They may be regarded as giving the minimal values for the axonic lipids.

### Summary

*Lipid development in grey and white matter was studied, from early fetal stages far into advanced age in man, and from birth to 70 days of age in cortex, whole brain and spinal cord in rat.*

*When correlated with histological data the results obtained in early development stages indicate a low concentration of lipids in the young, unmyelinated axon, consisting chiefly of lecithins and some cephalins while the small amount present of cholesterol is perhaps contained to a great part in some axonic coating. The lipid contents in the axon is yet larger than that in the nerve cell body, perhaps owing to the relatively greater surface of the former.*

*The predominance of lipids in the myelin sheaths is pregnantly illustrated by a diagrammatical comparison between brain and spinal cord during development.*

*The results on man should be useful as a normal material in studying the lipid pathology of the brain. The microscopically observable, physiological changes in the lipids in the myelination period and in advanced age are discussed.*

*Choline, pantothenic acid and vitamin A deficiency, respectively, as well as gammexane injections did not disturb lipid development in the nervous system of rat; in some cases but not others thiamine deficiency seemed to do so, the significance of which is difficult to determine.*

## Chapter VIII

### Lipids in Brain Tumours

From morphological aspects gliomas are very similar to normal fetal glia (147). Although they cannot be considered equivalent to fetal glia and still less to mature glia, e. g. owing to the lack of contact with neuronal elements, it was for the aims of the present investigation considered valuable to define their lipid pattern. During the period, the analyses were going on it was possible to obtain a limited number of tumours only, but never the less a fairly representative assortment of glial tumours of various origins can be presented. For the sake of comparison tumours from mesodermal supportive tissue, vascular and endothelial tissue were also analyzed, and so was normal pia-arachnoidea.

#### Experimental

Immediately following operative excision the tumours were frozen in dry ice in which they were stored until the analysis was performed. They were then thawed out, rid of any adherent grey or white matter as well as vascular and connective tissue and coagulated blood. In order to make certain that the correct tissue was isolated smears were stained and microscopically examined according to RUSSEL's method (146 a) prior to the usual subdivision and analysis.

The tumours were subjected to routine histological examination at the Histopathological Laboratories of the Caroline Institute, Stockholm, from which the biopsy reports emanate. The following may be emphasized concerning the separate kinds of tumours: All the *astrocytomas* were homogeneous and mature with the exception of III which was rich in vessels and fibres. The *ependymomas* and *malignant glioma* no. 3 were also rich in vessels. Necrosis was stated to be rather profuse in some of the malignant gliomas (nos. 1, 3, 4). Histologically the *gliomatous teratoma* was a pure glioma as was the *glioma of the nose*. Both these were homogeneous and mature but had been formalin treated for months. All the *meningeomas* contained an abundance of cells and vessels but no psammoma bodies; the *neurinomas* were ordinary tumours of n. acusticus. The haemangioma was an intermediary stage between h. racemosum and

cavernosum containing thrombi and fatty granular cells. Some vessels were filled with lipid containing cells (degenerated endothelium?). The skull bone was the site of *eosinophilous granulomas*. *Pia-arachnoidea* was taken from the dorsal side of the spinal cord of cow.

Since usually small quantities only of tumorous tissue could be isolated from the preparations the analyses are incomplete in many respects.

### Results

For results see table XI.

There are no noteworthy differences in the lipid composition of mature and immature *gliomas*. The individual values vary quite a lot but this is not surprising as regards tissues like these. The average phospholipid contents is about 10 %; free cholesterol about 1.6 %, total close to 2 %; cerebrosides 2-4 %, all on dry substance basis. If it could have been computed in all the cases the unidentified fraction probably would have varied most. The »diglyceride» fraction was small in the only fresh analyzed. In the glial teratoma, on the other hand, it was large, which partly may have been due to decomposition of glycerophospholipids during the formalin treatment, which probably also is the reason for the low phospholipid contents in the gliomatous teratoma and the nasal glioma.

The lipid contents in the *meningeomas* is practically identical to that of the *gliomas*, while possibly the *neurinomas* contain slightly less lipids. The *haemangiomas*, on the other hand, contain little phospholipids but they are very rich in cholesterol of which rather more than 60 % is esterified, in other words the same proportion as in the blood. It may be suspected that the histologically observable lipid cells in the vascular lumina predominantly contained cholesterol that had been absorbed from the blood. Naturally the lipid contents cannot be considered normal for blood vessels. The *eosinophilous granulomas* had much the same composition but here lecithins were strongly predominant in the phospholipids (approximately as in the blood).

In comparison to tumours the normal lipid picture of *pia-arachnoidea* is much poorer. The large unidentified fraction may be supposed chiefly to consist of neutral fat.

A larger amount of inositol could be extracted with chloroform from malignant *gliomas* than from *meningeomas*, but

the experiments are too few to permit the drawing of any conclusions. It was shown by means of paper chromatography that choline, ethanolamine and sérine were present in the lipid fraction of gliomas; phosphatidyl ethanolamine made up 70 % of the cephalins.

### Discussion

Probably the small amounts of lipids in the pia-arachnoidea must be assumed to be derived from its abundant supply of nerves and the numerous blood vessels which unavoidably contaminated the preparation. Such being the case the result implies that only minute quantities of lipids are contained in ordinary mature connective tissue. (Cf. FLETCHER et al. (63) who in connective tissue from rat (unknown source) found 0.26 % choline phospholipids on fresh substance basis, i. e. about 1.3 % on dry substance basis.)

Thus, it would seem, judging by the meningeoma analyses, as though formation of tumours in mesenchymal connective tissue (demonstrated in other cases also) increases the lipid contents of the tissue. Indeed, this is what could be expected as a result of the increased number of cells in proportion to the amounts of intercellular fibres. If the same reasoning holds for ectodermal supportive tissue the normal lipid contents in the glia should therefore rather be lower than that indicated by the glioma analyses. However, nothing can be said with certainty about this matter. Possibly it may be safe to assume that the lipid pictures of fetal glia and gliomas are approximately the same.

It was slightly unexpected to find essentially similar, both qualitatively and quantitatively, lipid contents in meningeomas and gliomas, just as it was to find glycolipids in both. BRANTE and SVENNERHOLM (28) have even found signs of gangliosides in one glioblastoma out of three. Moreover, the *proportions* of the separate lipids differ only little from those in vertebrate »grey matter» table VIII), especially if a correction is made for the presence of a certain amount of myelin in the latter. The whole picture is very strongly reminiscent of that in brain in very early fetal stages.

Thus, the general impression is that *the lipid combination*

in question is not specific for any special kind of cell in the nervous system with membranes, but a *fairly general cytoplasmic ingredient* both in rather undifferentiated cells and in a number of differentiated ones. Quite another matter is that the lipid combination may constitute a larger (adult central grey) or smaller (tumours, fetal tissue) portion of total solids. Its concentration should be low in the presence of abundant extracellular collagen (e. g. peripheral grey matter) and high where lipidiferous structures are strongly developing. The plasma membranes are characteristic representatives of the latter, and they surely ought to be abundant, just as cytoplasm is, in mature grey matter, judging by its extremely ramified cells and the resultant, enormous cellular surface.

The demonstrated correspondence between the lipid contents in glia and nerve cells is not so strange, considering their common origin. As regards the meningeomas one school (see 190) considers them to be of neuroepithelial origin. However, probably there is nothing to prevent mesodermal tumours from having a lipid contents like that of the meningeoma.

Some discrepancies in the tumour results from those of normal tissue must not be forgotten, however, in this connexion. Part of the cholesterol was constantly esterified in the tumours, sometimes very much so. In some cases, moreover, neutral fat seems to have been present. Both these »extra» lipids may, however, largely be products of necrosis — a not uncommon condition. BIERICH and LANG (15) have clearly demonstrated a similar effect of necrotization in another type of tumour (Jensen sarcoma).

### Summary

*The lipid contents was largely the same in gliomas and meningeomas. The major portion of the essential lipids consisted of phospholipids, but both cholesterol and cerebrosides were also present. The cholesterol was partly esterified, neutral fat was apparently present in some cases; but these two findings are assumed to be partly due to necrosis. Normal pia-arachnoidea contained negligible amounts of lipids in comparison to the tumours.*

*The combination of lipids found in glioma and meningeoma is composed like but is not as abundant as that in fetal brain and adult cortex; it may be a rather unspecific, general cytoplasmic constituent.*

*Haemangiomas in the brain and eosinophilous granulomas in the skull bone were characterized by the high contents of cholesterol, which exceeded the phospholipid level and to more than 60 % consisted of cholesteryl esters, probably contained in degenerated endothelial cells or other phagocytic elements.*

## Chapter IX

### Lipid Changes in Spinal Nerve during Wallerian Degeneration

Judging by histological results Wallerian degeneration of peripheral nerves, despite being a very complex process, was thought to supply some information as to the topical distribution of the lipids, the data on the changes in the various histological structures being fairly precisely determined.

#### Experimental

More than 1 year old rabbits of different breeds were used. Under ether anesthesia and sterile conditions the ischiadicus was cut as close to foramen ischiadicum as possible, the proximal end of the peripheral stump was dislocated in among the surrounding gluteal muscles, fascia and skin were sutured. Animals were killed at suitable intervals by blowing air into the marginal ear vein. Equal lengths of the ischiadicus — from the incision to the heel — were excised for analysis from the healthy and degenerated side. The healthy and the degenerated nerve was alternately excised first, and this was left lying in the muscles while the other was excised. The top 1-2 mm of the degenerated nerve often looked slightly inflamed and was removed, as was a corresponding portion of the healthy nerve. Then the epineurium was apparently completely removed by withdrawal with the aid of delicate tweezers, and the nerves were frozen in »dry ice«. From the time of death the whole procedure took 30-45 minutes. Each analysis included nerves from 2-3 animals. No signs were observed at any time of establishment of connection between the distal and peripheral stumps, nor were there any signs of infection. The animals seemed healthy all the time.

#### Results

In table XII the values on a total solids basis for degenerated nerves may be directly compared with the corresponding figures for the healthy side.

12 hours after cutting cholesterol and cerebrosides are unchanged, phospholipids slightly reduced. The reduction is wholly limited to the



KOH non-decomposable group, more precisely cephalins B, while sphingomyelins have increased. However, these changes are too small to be significant on the basis of a single analysis.

After 72 hours the reduction in phospholipids is more pronounced and accompanied with a slight cholesterol and cerebroside decrease. The KOH non-decomposable group of the phospholipids is further reduced, but now in all probability owing to sphingomyelin losses (lecithins were not determined but judging by results before and after 3 days they may be assumed to be unchanged).

After 6 days conditions are much the same, although the decrease in KOH non-decomposable phospholipids is less pronounced, cephalins B have even increased while a slight reduction in cephalins A has set in.

After 8 days the position as regards total phospholipids and cholesterol is similar.

13 days after the operation the changes are considerable. To be sure, total cholesterol is not much more reduced than in previous groups, but quite a lot of it is converted into the esterified form. About 50 % of the cerebroside have disappeared (N.B. without increasing the unidentified fraction). At this time cephalins A have gone down about 45 %, *cephalins B on the other hand have gone up markedly* and simultaneously sphingomyelins have decreased about 75 %; the net result being a slight loss of KOH non-decomposable phospholipids. In contrast to the great changes in the other groups lecithins still are almost uninfluenced.

In the 16 days group the loss in cephalins A and sphingomyelins is even more accentuated and so is the esterification of the cholesterol. Strangely enough there is no decrease in cerebroside worth mentioning. As before lecithins are unchanged.

On the 19th day the result differs from the preceding one by an apparent slight retardation in the sphingomyelin decrease attended by a considerable loss of cephalins B, whereby the entire group of KOH non-decomposable phospholipids has become strongly diminished.

This tendency is still more accentuated in the values after 23 days. Cephalins B have totally disappeared. For the first time during the progress of the degeneration the lecithins are markedly lower. The cholesterol is 50 % esterified and the cerebroside is reduced by half. Cholesterol fatty acids constitute a considerable portion of the unidentified fraction.

Briefly, the following general tendencies were observed towards *lipid changes in relation to other solids*.

- 1) A decrease in the concentration of all the essential lipids. It was noticeable but slight during the first week and almost 50 % after 3 weeks.
- 2) A slight initial drop in the cholesterol level followed by progressive esterification but only slow disappearance.

Thereby *free* cholesterol vanished slowly in parallel with other lipids.

- 3) Progressive reduction in cerebroside.
- 4) Progressive loss of cephalins A and (apart from 1st. day) sphingomyelins during the first 16 days, then the former remained constant and the latter to some extent regenerated. The changes in cephalins B were the »negative» of the sphingomyelin picture.
- 5) Lecithins remained unchanged, at least during the first 19 days; if anything, »diglycerides» decreased.

But not only the lipids are affected by the degenerative process. The losses observed may wholly or partly be merely relative and due to an increase of other solids caused by cellular proliferation and invasion. The adopted method of computation tells nothing of the *absolute lipid changes per nerve*. The only approximate figures for the weights of the excised nerve portions (the rinsing of which may have entailed incalculable losses) were therefore used to roughly compute the absolute quantities of lipids per nerve. The quantity of a certain lipid in the degenerated nerve was then converted into a percentage of the amount of the same lipid in the nerve on the fresh side. The standard deviation must here naturally be considerable and, judging by the figures for water contents and non-lipid solids, the lipid values in the 13 days degenerated nerve should be considered as too high, those in 16 days degenerated nerve as too low. If this is taken into account *the picture in several respects becomes the same as that on a total solids basis*. However, it is revealed that already after 13-16 days the lecithins begin to decrease (fig. 5) and that after 23 days this decrease amounts to 50 %. Never the less, it is obvious that the loss of choline phospholipids all the time chiefly is equivalent to a drop in sphingomyelins. The changes in the cephalins B group drastically contrast to those in the other phospholipids, especially the sphingomyelins.

From more general aspects the curve in fig. 6 demonstrates agreement in the degeneration of total phospholipids, cholesterol (considered in its normal free form) and cerebroside:

% of normal amount

Figure 5.

Diagram showing the changes of the phospholipids in % of contents per normal nerve during degeneration.

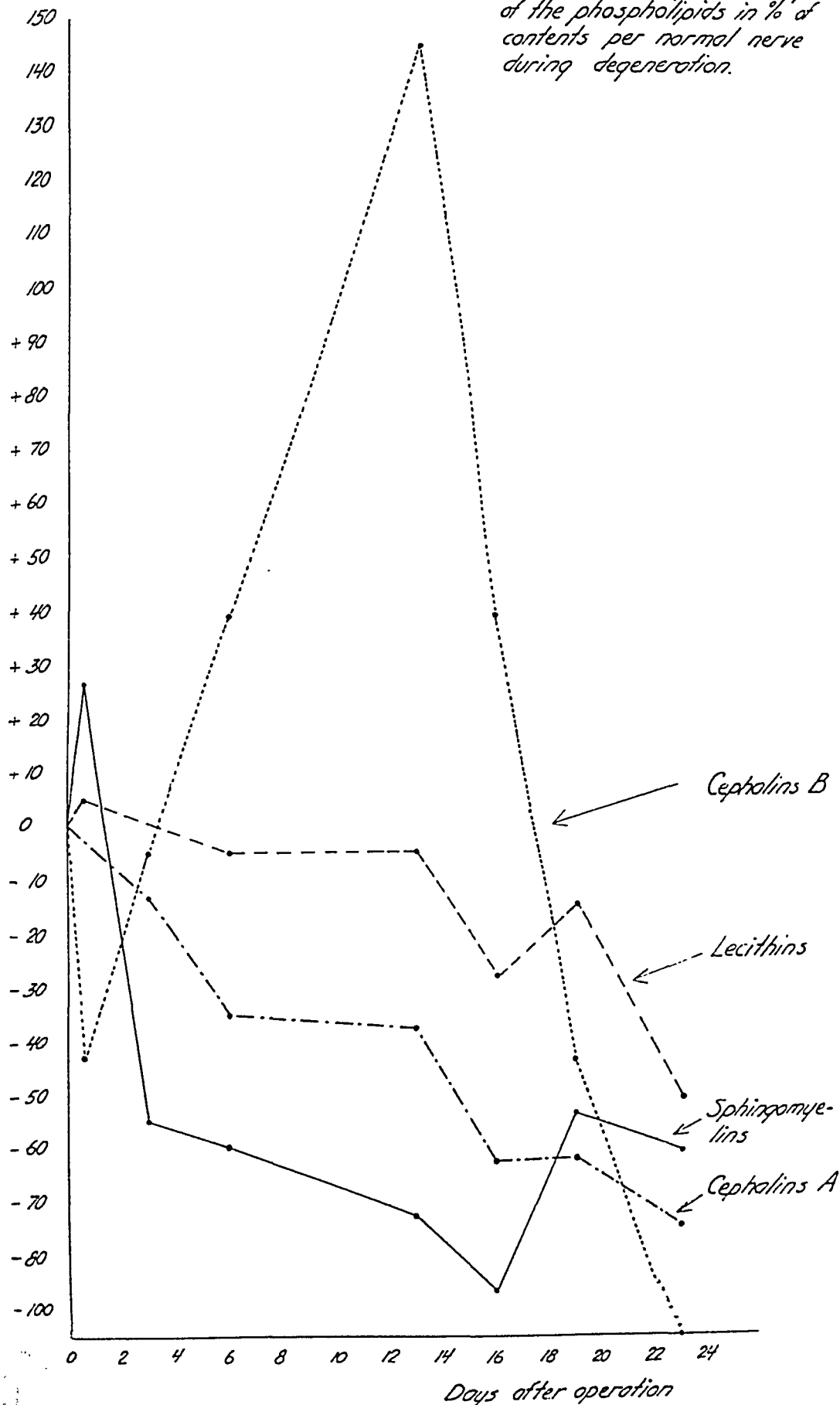
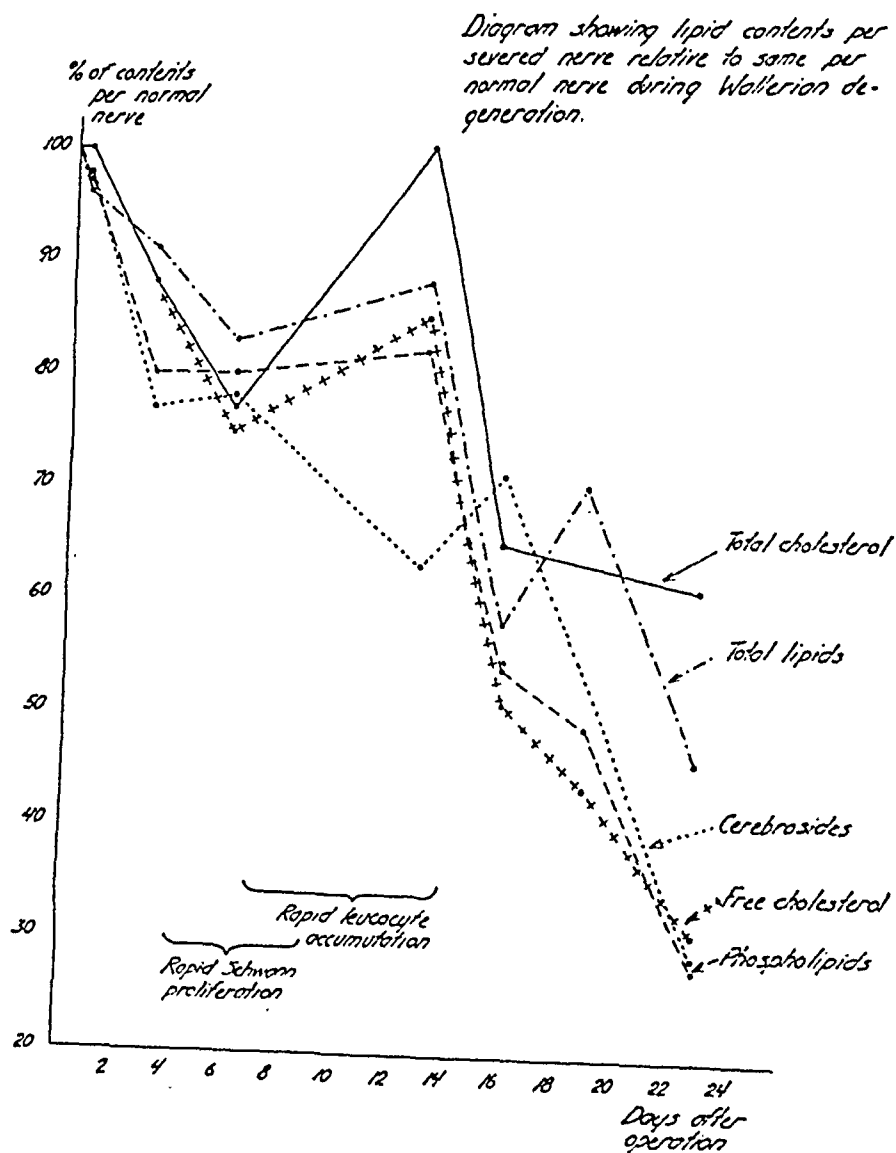


Figure 6



a short steep decrease during the first 3 days, then relative constancy, which after 13 days passes into a new steep drop. However, here the author would again stress that it was impossible to get absolute equivalent materials from the small animals and that individual differences in the rate of the degenerative process seemed possible, judging by macroscopical inspection. With this reservation only was it considered justified to present the curves just described of the absolute lipid changes per nerve.

### Comparison with Earlier Investigations

Hitherto only few chemical investigations on lipid changes under Wallerian degeneration have appeared. NOLL's results (131) as to the decrease in protagon (see p. 20 for composition) during nervous degeneration are probably the earliest. It is interesting for the investigation in question that only 3-4 % of the protagon disappeared per horse nerve during the first 8 days. After 14-23 days 40-50 % had vanished, after 28 days practically 100 % (horse, dog). Thus, considering that NOLL's test animals were larger, there is good agreement with the present author's cerebroside and sphingomyelin results.

In cat MOTT and HALLIBURTON (80) among other things studied the P fraction which still could be determined in ischiadicus nerves, subjected to Wallerian degeneration for varying periods, after soaking for 21 days in 5 % HCl. This P fraction was wholly or mainly thought to be derived from phosphorized fat. According to present knowledge some phospholipids (e. g. acetal phospholipids) should have been released or decomposed during the acid treatment; the P results therefore not including all phospholipids. The gravimetric P method employed probably also was rather too insensitive for experiments of this type. However, the authors demonstrated a progressive P decrease in solids, slow during the first 6 days and at most amounting to 20 % of the normal value. Thereafter the decrease became more rapid, during 8 days the loss was 55 %, 10 days 70 %, 13 days 80 %, 25-27 days almost 100 %. Zero P was present during regeneration for 44-60 days while after 100 days 80 % of the normal value had been attained. The zero values cannot correspond to actual conditions as regards phospholipids, for the abundance of cells in the regenerating nerve during the stages with zero P reasonably must contain phospholipids, even if the lipids in the myelin sheaths have disappeared completely (hardly after 29 days). MOTT and HALLIBURTON's normal values and early degeneration values for P, converted into phospholipids by multiplication with 25 (27.5 % and 22.5 %, respectively), agree very well, however, with those of the present author. This fact may indicate that lipoprotein compounds must be physically deranged and perhaps broken up to render the lipids susceptible to the disturbing decomposition or liberation caused by the HCl treatment, and that this requirement is fulfilled by the degeneration.

MOTT and HALLIBURTON also studied the choline contents in the blood during regeneration, after 4 days finding an increase to a maximum on the 8th. day with a continued high level until the 13th. day and then a rapid decrease to normal values. However, these results were obtained with rough chemical methods and with probably unspecific physiological performances, and therefore they need to be checked by modern methods. As regards time, however, they agree well with the rapid decomposition of phospholipids in ischiadicus of cat and would therefore, as supposed by these investigators, represent a release during degeneration of choline from phospholipids. In my own experiments

with rabbit ischiadicus the biggest drop in choline phospholipids occurred between the 6th. and 16th. day, although already earlier the sphingomyelins decreased considerably.

In more recent years MAY (121, 122, 123) has carried out a series of investigations into changes during Wallerian degeneration in ischiadicus from dogs and rabbits. He determined various P, N and S fraction and total cholesterol, using methods which, judging by the double determinations, entailed fairly large errors. A primary alcohol and ether extract of fresh nerve was reextracted (apparently without heating) with ether-benzene  $1/1$  and supplied the lipid fraction. Owing to solubility properties this reextract would include only part of the sphingomyelins, the remainder being included in the subsequent alcohol extracts after washing the residue of the original extract with water.

If the total amount of P in MAY's lipid and alcohol fractions is converted into phospholipids his normal values will be on level with mine. Therefore, the changes in these fractions, especially, are of interest for the present investigation. As compared to normal values P, calculated on a dry substance basis, in the lipid and alcohol fractions changed during the degeneration as follows:

in 7 days by — 0.4 % and — 8 %, respectively

" 14 " " — 47 " " — 7 " "

" 21 " " — 54 " " + 9 " "

" 33 " " — 67 " " — 67 " "

" 100 " " — 90 " " — 70 " " , and from then

onwards scarcely at all. These figures refer to rabbit. In dog N in the lipid fraction behaved much like P above; in the alcohol fraction, on the other hand, it increased, which if anything was due to non-lipid substances (purines?) difficult to dissolve in water and not to lipids. Largely, MAY's results mentioned up to the present may be said to agree with mine. After 100 days every trace of myelin rests had disappeared; then P in the lipid (and possibly the alcohol) fraction should be a measure of the phospholipids in the proliferated Schwann cells. The approximate value, 2 % of total solids, is probably on the low side, however, since large amounts of ordinary connective tissue are present besides the Schwann cells (cf. lipid contents in pia-arachnoidea, table XI).

The agreement is not so good between MAY's results (122) for total cholesterol (gravimetric digitonin method) and my own. During degeneration for 7-35 days (rabbit ischiadicus) MAY consistently finds cholesterol, in % of total solids, increased in proportion to normal values and from the very beginning by not less than about 25 %. However, MAY looks upon the increase as being merely relative and due to a greater loss in proportion to cholesterol of other solids, for on a fresh substance basis cholesterol decreases progressively. This can hardly be the whole truth, however, his own results for solids apart from cholesterol can scarcely explain the observed early cholesterol increase

in dry substance as being other than at least partly real. MAY's normal cholesterol value in ischiadicus on a total solids basis, 7.8 %, is considerably lower than the author's, although his values after degeneration, about 8-12 %, are on my level. It may be wondered whether some phase of MAY's method reduced the determinability (extractibility? digitonin precipitability?) of cholesterol in normal material or increased it in degenerated.

### The Relation between Structural and Lipid Chemical Changes

When the experiment originally was planned the train of thought was the following. After degeneration for 8 days axons are practically absent while sheaths are deranged but only slightly resorbed. The deviation from the normal lipid picture would then mainly consist of axonic lipids. As mentioned the result was that only small amounts of lipids vanished and of these merely such as in chapter VI were characterized as typical of myelin sheaths. This must imply that either the axonic lipid picture is different to that assumed in chapter VI, or otherwise the histologic disappearance of the axons from the nerve fibres does not necessarily entail that their chemical components decompose or leave the tissue. In the latter event the mechanism on the part of the lipids might merely be an absorption in the disorganized sheath lipids. If no decomposition of the axonic lipids took place locally in the axon prior to their release to the myelin, the axonic lipids would in the latter mechanism be attacked relatively late, probably only on having reached the surface of the sheath and become accessible to decomposition by external forces (Schwann cells, leukocytes). This in fact seems to be the case as regards lecithin. This supposedly axonic lipid began to decrease only during a relatively late degenerative stage, approximately at the peak of the leucocyte invasion. If a substance as generally metabolizable as lecithin had been accessible to decomposition from the very beginning, its late disappearance would be difficult to explain. An alternate explanation would be that the proliferating Schwann cells compensated for or rebuilt decomposed lecithin.

In interpreting the results of the degeneration experiment at least 2 periods must be distinguished: 1) the preleucocytal,

and 2) the leucocytal. During the former the changes are released wholly by means of the cellular elements inherently present. The latter, however, does not remain constant during the period, for Schwann cells swell up already during the first days and proliferate as from the 4th. day, with maximum between the 6th. and 9th. day. Thus, period 1) comprises a preparatory stage with moderate changes only in the cellular elements, = the first 3 days, and a later stage characterized by lively multiplication, = 4th. until about the 8th. day. During the period the active enzymes those present in axons, sheaths and Schwann cells, and, notably, without the normal stimulus from nerve impulses. The very earliest changes may be assumed to correspond the normal chain of events at rest. The 12-hours lipid results would indicate a charge of the nerve fibre with sphingomyelin attended by consumption (choline esterification?) of »cephalins B» (at least in part, see p. 42, these probably are sphingolipids). Totally, the KOH non-decomposable group of phospholipids decreases, a result agreeing with the experiences of the autolysis experiments. The 12-hour changes must be shown to be reproducible, however, to be considered valid. Matters are more clear-cut as regards some changes during slightly later stages. Then there doubtlessly is a decrease in sphingomyelins and, owing to the simultaneously increasing values for Cephalins B, among which any of choline freed sphingomyelins will be grouped it seems to be caused simply by a release of choline. Possibly it was this choline MOTT and HALLIBURTON detected in blood during degeneration, at any rate it was hardly choline from lecithin as these authors supposed. The small simultaneous drop in cholesterol and cerebroside suggests that during the first period »sheath typical» lipids in general partly vanish from the nerve fibre. Apparently this takes place through the interaction of enzymes in the myelin sheaths or Schwann cells — enzymes which during physiological conditions perhaps catalyse the reactions in the opposite direction (cf. e.g. the high cephalins B values simultaneous with low sphingomyelin values during myelination, a state disappearing at maturity, p. 145). The constancy of lecithin during the first period may, as mentioned, be the result of a less open localization as in or



near the axon; the reduction in cephalins A may be one of phosphatidyl serine. From these points of view the results of the degeneration experiments are not incompatible with the picture drawn in chapter VI of the lipid distribution in axons and myelin sheaths, but the aspects do not convincingly support the theory.

The most rapid rate of leucocyte infiltration takes place from about the 8th. to the 14th. day, thereafter practically no new leucocytes arrive. Judging by the results of the analysis but insignificant amounts of lipids vanish during this period which, therefore, rather implies a charging of the phagocytes with nerve fibre constituents. During this process cholesterol is partly esterified and choline released from sphingomyelin, which perhaps is nothing but a continuation of the previous process. After the 13th. day the lipids begin to vanish at a seemingly equal rate for all types except some of the cholesterol which, being esterified, remains longer, and possibly some unidentified lipids (fatty acids bound to cholesterol deducted). The latter should then not contain any increased amount of triglycerides, for the »diglyceride» values, when the former would be present, decrease fairly parallelly with other lipids. Consequently *there is no proof of the generally accepted view that neutral fats are formed from phospholipids during Wallerian degeneration.* The Sudan staining, isotropic substance that is released from the myelin sheath and transported in phagocytes probably contains both unchanged myelin sheath lipids and cholesteryl esters as well as partial decomposition products such as diglyceride and lignocerylsphingosine phosphoric acid esters, ceramides, fatty acids. All these exist in an unorganized and undispersed state, which accounts for their properties in polarization microscopy (159) and fat staining.

#### Summary

*During Wallerian degeneration the most marked feature as regards lipids before the leucocyte invasion is a release of choline from sphingomyelins. At the same time there are small losses of cholesterol, cerebrosides and cephalins, while lecithins remain unaffected. The only lipid group to increase is »cephalins B». Its increase parallels the decrease in sphingo-*

*myelins and is therefore assumed to consist of ceramide phosphoric esters. If during the early stages the myelin sheath is assumed to protect the axonic lipids and these are secondarily released into the myelin, the results are compatible with the picture given in chapter VI of the lipid contents in axons and myelin sheaths.*

*After the leucocyte infiltration has been completed most of the lipids are removed at approximately the same rate. Cholesterol is largely esterified and remains longer. There are no signs of neutral fat formation.*

## Chapter X

### Lipid Changes in some Demyelinizing Diseases

In man there exists a group of diseases which have in common that they selectively destroy the myelin sheaths without correspondingly affecting the nerve cells or their axons. The most common and best known are multiple and diffuse sclerosis. I have had the opportunity of studying three cases of the latter fairly rare condition, and these are the subject of the present chapter. For comparison one case is included of epidemic encephalitis which besides demyelination also exhibits considerable destruction of axons.

#### Material

*Diffuse sclerosis.* All three preparations were stored in formalin. The first case has already been published in a paper by KAIJSER and LUNDQUIST (91) in which the chemical analysis and comments are mine. The second case will soon be published more fully by BRANTE, KAIJSER and FRÄNKEL. The third case had been preserved in formalin for as long as 11 years.

In all the 3 cases the ›white matter‹ was greyish, hard and tough. Typically changed but not disintegrating pieces were taken. The grey matter in the spinal cord was carefully removed. After dipping in distilled water the materials were sectioned and analyzed in the usual manner.

*Encephalitis.* 59 years old woman. Died 1 week after beginning of symptoms. Autopsy one day after death. Consistency of brain was soft and resilient, the cortex was locally disintegrating. No formalin treatment.

#### Histological data

Case I (see 91). The cortex was largely normal with the nerve cells preserved. In the marrow severe disappearance of myelin sheaths, locally axonic do. and profuse embryonal and pathological glia with epitheliform cells; by clinical and histological data classified as KRABBE's disease. Case II. Largely the same picture. The epitheliform pathological glia cells still more abundant. KRABBE's disease. Case III. SCHILDER's disease. Histological findings equivalent to those in cases I and II.

## Results

**Diffuse sclerosis.** The results of the first case were originally grouped in a manner different from the one used here. They were later completed with a determination of KOH releasable phosphorus (lecithins+cephalins in (91) were calculated on the basis of glycerol determination) and in their new form the values are presented in table XIII. a) and b) signify separate specimens from two different parts of brain white matter. The table also gives the figures for case III, while case II will be published separately and here will be discussed only briefly and correlated to the others. In considering the results every regard must naturally be taken to the formalin treatment.

**Case I.** Most of my previous statements concerning this case still hold (cf. the paper by KAUSSER and LUNDQUIST). Thus, the lipid contents in the *cerebral cortex* is approximately that to be expected normally according to the age and 1 year of formalin preservation (cf. fig. 2 and table VI). In white matter, however, the values for all the separate lipids are much lower than normal in comparison to other solids, this is especially the case concerning central white matter for which the values are merely  $\frac{1}{3}$ rd. of normal; here the values are even lower than in grey matter, in spinal cord white matter, on the contrary, they are about the same as here. Furthermore, the proportions between the various main lipid groups have shifted towards those in grey matter, i. e. cholesterol and cerebroside are relatively more reduced (mutually to approximately the same extent) than phospholipids. The KOH non-decomposable fraction of the latter seems relatively little reduced, cephalins most.

**Case III.** Owing to the excessive duration of the formalin treatment the results are more difficult to interpret. The figures for grey matter do not differ significantly from those in table VIII for cortex of adult woman, formalin-preserved for 8 years. The white matter values do differ, however, just as in case I the essential lipids are reduced by about  $\frac{2}{3}$ , the phospholipids as much, proportionately, as cerebroside and cholesterol. This may, however, be a result of the longer formalin treatment — a fact suggested by the uncommonly high unidentified fraction.

*Case II.* This material (from about 1 year old child) had been preserved in formalin for a short period only (a month or so) and is therefore to be considered more suitable for analysis than those from cases I and III. In most essentials the results agree with those described above. The cephalin fraction was, however, still more reduced in relation to the other phospholipids and the white matter was changed to about the same extent in the spinal cord and the brain.

**Encephalitis.** This condition involves abnormalities not only in the marrow but also in the cortex. The water contents are about normal; non-lipid solids have increased considerably in the marrow and possibly decreased a little in the cortex; the chief changes are to be found in the lipid fraction.

In the *cortex* the total amount of chloroform extractible solids is if anything slightly increased, and it turns out that the increase is derived from the unidentified fraction, the concentration of which is thrice normal. The phospholipid and cerebroside values are on the contrary distinctly lower than usual, which may imply that decomposition products from these groups are contained in the unidentified fraction; but they can hardly make up all the increase of the latter group. Nor can neutral fat which seems to be totally absent. Cholesterol seems to occur in approximately normal concentration but partly in bound form — another indication of lipid decomposition, or perhaps phagocyte invasion.

The changes in the lipids of the *marrow* are not less marked. In this connexion it must be remembered, however, that the relative increase in non-lipid solids entails some non-existent decrease in lipids on the basis of total solids. But even if expressed in percent of fresh substance the reduction of the lipids is very considerable, viz. about 35-40 % of cholesterol, cerebroside, KOH non-decomposable phospholipids and choline phospholipids. Just as in the case of cortex the unidentified fraction is higher than usual and this increase is not caused by glycerides.

#### Discussion

Already on an earlier occasion I have emphasized the similarity between the white and the grey matter in the lipid picture of case I and written (translated from Swedish):

»On comparing the values for white and grey matter it appears that there is a remarkable similarity between white matter in the spinal cord and grey matter in the cerebrum. In diffuse sclerosis the absolute lipid contents in brain white matter is lower than in grey. But here also the mutual proportions of the separate lipids are more like those in normal cortex than in white matter.

Since in the early stages of development cerebral cortex and marrow contain almost identical amounts of lipids, the observations mentioned above may be interpreted as an early incapacity of differential generation of white matter (=inadequate power of myelinization), or as a selective disappearance of the lipids in the myelin sheaths, caused by the pathological process. The result, whatever the reason, being that the marrow will consist of a matrix with infiltrations of substitutive tissue. This matrix is similar to the one normally present before the beginning of the myelin sheath development.»

The results from the new cases of *diffuse sclerosis* in conjunction with observations of glioma and various developmental stages of the human brain support my previous statement. It must be emphasized, however, that the substitutive tissue histologically only partially was identical to fetal glia. It to a large extent also consisted of accumulations of hypertrophied glial and adventitial elements, often polynuclear and filled with lipid material which apparently was of myelin or premyelin nature (their interior slightly Sudan staining according to GELLERSTEDT and erythrochromic according to FEYRTER and consequently partly consisting of glycolipids, cf. chapter XI). Finally, some more or less normal myelin sheaths were observed as well as degenerated axons and some fibres in apparent Wallerian degeneration, the picture in the white matter therefore in fact being very composite. Consequently the materials cannot supply the information hoped for, viz. the changes given by *pure* demyelination. Some conclusions can be drawn, however, provided that (which seems perfectly justified) the changes are regarded as a cytoplasmic increase at the expense of a loss in myelin sheaths. It then turns out that the cytoplasm (glial, axonic)

as compared with the myelin sheaths probably is poor in lipids, especially such belonging to the groups cerebrosides, cholesterol and cephalins. This is in good agreement with the observations in chapters VI-IX.

*Encephalitis* being a more acute process than diffuse sclerosis and affecting the whole neuron, it is interesting to compare the two. In encephalitis the *cortex* was involved in the process, but the nature of the lipid change was unclear since it was most pronounced in the unidentified fraction. In the *marrow* the process had resulted in equivalent losses of cholesterol, cerebrosides and KOH non-decomposable phospholipids, which, according to the conclusions in chapter VI, is compatible with demyelination and agrees well with the findings in diffuse sclerosis. So does the relative increase in lipids of KOH decomposable phospholipids and probably on the same grounds (cellular infiltration). Thus, *in encephalitis the lipid changes in the marrow were largely similar to those in diffuse sclerosis* but quantitatively much smaller; more rapid, however, when the durations of the two processes are compared.

The results obtained are hardly such as to permit any more significant conclusions to be drawn as to the origin of the conditions in question. The possibility that lipolytic ferments are the active agents in the marrow-sclerosing processes has been discussed (119); the fact that no lipolytically active contagion has been demonstrated in the brain has been given as a reason against an infectious etiology (11). The epidemic form of *encephalitis* must surely be infectious, so *in this case it must be virus which directly or indirectly causes a loss of lipids* from the brain similar to that in sclerosis.

The present author had rather expected that some special lipid would show itself selectively much attacked in diffuse sclerosis. It cannot be said that such actually was the case except perhaps as regards cephalins, especially cephalins A, which were most reduced. The formalin treatment makes interpretation difficult, however, so it is essential that fresh material also is studied.

In contrast to the findings in diffuse sclerosis other degenerative processes in the peripheral and central nervous system, e.g. Wallerian degeneration, anaemic degeneration

(162) and general paralysis (162), show an esterification of cholesterol, a slow removal of the same lipid relative to other sheath lipids and an increase of unidentified fraction. Therefore the sclerotic process might be assumed to include a more effective removal of degeneration products. This, however, is not borne out by the histopathological picture which, according to, among others, SCHOLZ (154), rather suggests insufficiency of the glia function as regards »Abbau». He emphasizes the accumulation of myelin sheath staining lipids in gliogenous cells. My own staining results are in complete concord with SCHOLZ' experiences. The chemical findings are well compatible with a disordered glia function.

### Summary

*Under the assumption that the lipid distribution between the cytoplasm and the myelin sheaths is the one suggested in chapters VI and VIII there is a far-reaching correspondence between the chemical findings of lipid changes and the histological experiences of demyelinization and cellular infiltration into the marrow in diffuse sclerosis and acute encephalitis.*

*Some discrepancies between the lipid changes in diffuse sclerosis and those associated with other degenerative processes in the nervous system are discussed and are assumed to be connected with a disordered glia function.*



## Chapter XI

### Comparisons between the Chemical and Recent Histochemical Findings as regards the Topical Distribution of Lipids in the Nervous System

The more exact knowledge of the lipid distribution in the nervous system, which in the author's opinion is offered by the investigations described, should have a number of consequences for the interpretation of the patterns obtained by histological methods. Down the years many histochemical lipid analyses have been attempted, but few of the results have stood up under careful examination. The author has no intention of systematically scrutinizing such methods.

Here some recent methods will be briefly discussed, the principles of which seem to be fairly well founded. The experiences won with these methods will allow further conclusions to be drawn from my own results.

#### Feulgen's plasmal reaction (58)

Under special conditions fuchsin is specifically reduced by fatty aldehydes, giving rise to a purple colour. In the nervous system this dye stains the myelin sheaths while unmyelinated parts of the cortex remain practically colourless. Another reaction, with 2,4-dinitrophenylhydrazine (2), which has been demonstrated to behave similarly versus plasmalogens, also is selectively positive for myelin sheaths. Consequently the plasmalogens seem to be almost exclusively located in the myelin sheaths. *Plasmal* has been found (59) to make up 8-10 % of phospholipids in brain. The histologically discernible myelin sheaths in the whole brain probably amount to about 25 % of the total mass and their phospholipids to more than 50 % of total phospholipids. Thus, probably 25-30 % of myelin sheath phospholipids are *plasmalogens*. My

own investigations showed that the concentration of ethanolamine phospholipid and total phospholipid in the myelin sheaths in white brain matter is about 3.9 % and 15.3 %, respectively of fresh tissue, i.e. ethanolamine phospholipids amount to about 25 % of myelin sheath phospholipids. The correspondence between FEULGEN's figures and my own would seem to suggest the possibility that *most or all of the ethanolamine phospholipids contained in myelin sheaths are present in the form of plasmalogens.*

#### Feyrter's olycolipid staining (60 a)

Under FEYRTER's special conditions thionin induces metachromasia for red in some otherwise non-metachromatic tissue structures. Judging by extraction experiments the metachromatic tissue substance in question is of lipid nature and by in vitro experiments with purified lipids probably some glycolipid. The method has been criticized (PISCHINGER (138)), the positiveness being attributed to formalin from the fixation adhering to some tissue elements which normally would be negative. Be that as it may, yet there are, as mentioned previously, some lipids which in a purified state induce metachromasia already without formalin treatment.

Using FEYRTER's procedure, the present author tested a series of purified lipids, finding pure cerebroside and gangliosides strongly positive; lecithin, hydrolecithin, phosphatidyl serine, phosphatidyl ethanolamine, »diphosphoinositide» and sphingomyelin negative. The result was the same with and without preliminary formalin treatment. Some of these substances had earlier been tested with the same result by FEYRTER (60 a), who in addition obtained negative results with cholesterol, neutral fat and some fatty acids and lastly with cerebroside sulphuric acid. The latter rather confusing finding might indicate that to react one end of the hexose molecule in the cerebroside must be free.

As regards the nervous system, FEYRTER has found that myelin sheaths give a strongly positive reaction, which is very easily explained by their great abundance of cerebroside, revealed by chemical analysis. But axons also induce erythrochromia even if it be weak; not weaker, however, than for the author to recommend the method as especially suitable for tracing the paths in tissues of unmyelinated fibres. The axon staining is not so easily explained. Possibly it may be an instance of unspecific reaction in the sense of PISCHINGER, but

the presence of glycolipids inside the axon is not out of the question. My cerebroside analyses demonstrate the presence of glycolipids in unmyelinated fibres also, but their localization is unknown and may be in SCHWANN's cells and the axonic surface just as well as in the inner of the axon. FEYRTER's result may suggest the latter localization.

Does the axon contain cerebroside or ganglioside? By means of analysis according to KLENK (102), BRANTE and SVENNERHOLM (28)) consistently found a small but significant ganglioside content in different unmyelinated nerves. In white matter gangliosides cannot normally be demonstrated by this method, but this may be due to disturbing effects derived from the cerebroside simultaneously present in large amounts. In diffuse sclerosis, where the myelin largely has disappeared without a corresponding reduction in axons, BRANTE and SVENNERHOLM could demonstrate conclusively the presence of gangliosides in brain marrow. The localization of the gangliosides in cortex, finally, may fairly safely be attributed to the nerve cells. In TAY-SACH's disease the nerve cells swell enormously because of lipid accumulation. KLENK (101, 97) has demonstrated that the deposited lipid is ganglioside; FEYRTER (60) has demonstrated pronounced erythrochromia of the abnormal cell contents. The lipid deposition is assumed to be related to and possibly start with an increase of the lipid structure normally occurring at the axonic base in the nerve cell, later it is accumulated in the glia also. FEYRTER has found the erythrochromic lipid also in peripheral neurogenic cellular elements at the terminal ramifications of the nerves. While they naturally may have been formed in loco as a sign of a general metabolic disturbance in a specific cellular system, they could very well have got there via the axons which then would form the normal route for gangliosides which are produced in nerve cells and overproduced in TAY-SACH's disease.

The above conclusions may of course be considered prematurely drawn. In the opinion of the present author, however, the histological and chemical facts mentioned unanimously indicate that *the gangliosides in the nervous system are components in the neurocellular cytoplasm, chiefly,*

in all probability also of the part forming the axon. Furthermore, they may pathologically and to a lesser extent also normally be contained in the glia cells. If in addition cerebrosides are contained in the neurocellular cytoplasm is more difficult to say — the investigations of fetal stages, however, suggest that if so their concentration is low.

#### Alsterberg's staining method for unsaturated phospholipids (3, 4, 5)

By means of a fairly cumbersome method involving AgCN precipitation from a JCN-AgClO<sub>3</sub> solution in the presence of unsaturated fatty acids and subsequent conversion of the deposit into AgS, ALSTERBERG maintains that he is able specifically to stain colloiddally dispersed unsaturated fats, and consequently *unsaturated fatty acid containing phospholipids* in the state in which they normally appear in the tissues. He basis his theory on a number of experimental investigations both on tissues (staining in connexion with fractionated lipid extraction) and purified lipids. Largely the method seems to be well founded, although, owing to the peculiar structure of tissues, many side-effects foreign to lipids remain to be eliminated.

In the nervous system ALSTERBERG reports the following reactions: nerve cells, dendrites, glia cells and axons in myelinated fibres, as a rule strongly positive; unmyelinated axons (e. g. in spleen nerves and cortex) and myelin sheaths, only weakly positive. Hence and because of experiences from extraction experiments, etc., ALSTERBERG states the following to be the *main* phospholipids: in nerve cell protoplasm lecithins; in axis-cylinders lecithins; in myelin sheaths traces of lecithins, little if any other unsaturated phospholipids; in glia cells cephalins. An especially interesting finding of ALSTERBERG is that myelin forms develop chiefly from the axis-cylinders. It has long been known (76) and is easy to confirm that the unsaturated phospholipids (according to my own experience especially lecithins, phosphatidyl ethanolamine and the possibly existing diglycerophospholipid mentioned on p. 47) generate myelin forms most easily.

As regards the predominantly cytoplasmic localization of

unsaturated phospholipids my own findings bear out ALSTERBERG's, just as they did with determinations of the iodine number (p. 120); ALSTERBERG even considers his method a »microscopical» iodine number determination. There may be a discrepancy, however, with respect to spleen nerves as well as other unmyelinated nerve fibres (e.g. nerves under fetal development), in which I find the lecithins contents fairly high; ALSTERBERG on the contrary obtains only a weak colouration. This discrepancy may be due to higher degree of saturation of the lecithins in these fibres or, for unknown reasons, a too weak response from ALSTERBERG's method as applied to naked axis-cylinders. Just as I do, ALSTERBERG finds signs of small amounts of lecithins in the myelin sheath also and therefore its exclusive localization to the axon (see p. 122) seems less likely. A greater accumulation of lecithins in the axons of myelinated fibres as suggested by ALSTERBERG is by no means incompatible with my own results (cf. p. 122). ALSTERBERG's preponderance of cephalins in glia cells does not agree with my findings in glial tumours, diffuse sclerosis, etc. This may be a manifestation of the pathological condition; ALSTERBERG's conclusions on a solubility basis are on the other hand open to criticism, since very little is known of lipid linkage in various cells.

#### Concluding Discussion

The following final tentative picture of the lipid distribution in the mammalian neuron is based on the available histological and chemical data.

*The cytoplasm* of cell body and branches contains unsaturated phospholipids of the lecithin type (1.1) as well as ethanolamine cephalins (1.1); possibly both these are more concentrated in myelinated axons. The cytoplasm also seems to contain gangliosides (0.5), whereas ordinary cerebroside probably are lacking. If cholesterol belongs here it may be located mainly in a surface layer.

*The myelin sheath* lipids are composed of cholesterol (8.4), cerebroside (9.8), sphingophospholipids (4.8), phosphatidyl serine (4.2), plasmalogens (3.9), and probably a small amount of lecithins (2.4).

other components in the materials exposed — are tentatively subgrouped in axon (A) and sheath (S) parts.

Although the primary division of the lipids into axonic and sheath groups hence may be assumed to have been largely performed, their internal position in the structures in question remains to be determined.

The *axonic* lipids may be localized 1) in a true sheath belonging to the axon (in myelinated fibres, presumably inside the real myelin sheath), 2) a special condensed surface coat of the axis-cylinder (equivalent to a plasma membrane), 3) the fibrillary structures in the axon or 4) interfibrillarly in the axoplasm.

There is much evidence in support of the existence of *alternative 1*). As early as 1913 GÖTHLIN (76) clearly showed the presence of oriented lipids in the surface layer of or as a cementing substance around the axons in some evertbrate nerves, formerly thought to be unmyelinated, among them claw nerves of Homarus (lobster). He substantiated the belief that cholesterol is of especial importance in this lipid layer. In recent years many works, particularly those by SCHMITT and coworkers (150, 151, 152), have shown that most evertbrate as well as many so called unmyelinated vertebrate nerves have around their axons thin sheaths with optical properties equivalent to those of vertebrate nerve myelin sheaths. However, these thin »axon sheaths» make up only some few % of the diameter of the axis cylinder and their protein component is relative to their lipid do. much more important than it is in ordinary myelin sheaths. Consequently the amount of sheath lipid would scarcely be more than fractions of one per cent of fresh nerve fibre. At least part of the lipids I have found in lobster nerves must be localized in the »axon sheath», but which and how large a part is hard to say. As regards insect nerves, in which RICHARDS (144, 145) has demonstrated the presence of similar sheaths around the axons, PATTERSON et al. (136) maintain that their lipid values for bee brain (see p. 116) correlated with RICHARDS' optical results prove that »a large part of the lipids are located in the sheaths of the bee's nerves but presumably an unknown fraction would be obtained from the nerve cells and fibres themselves.» RICHARDS

presents some evidence for the presence of phospholipids in the sheath.

Thus, it is clear that to the structure usually called the axon there may belong a lipid-containing sheath which in fact from many points of view has proved to be equivalent to the ordinary myelin sheath. This sheath has been shown to be able to envelop the axon continuously as well as the nerve cell belonging to the latter and its structural similarity to the *plasma membrane* of cells in general has been emphasized (76, 201). The »axon sheath» and also the myelin sheath could be an intense magnification of this plasma membrane. In several respects, however, the comparison misses, it has for example been demonstrated (already NAGEOTTE (129), later by BEAR et al. (14)) that the *evertbrate* »axon sheath» really is separated from the axon by a layer of thin cells, probably analogous to the Schwann cells of vertebrate nerves. For this reason comparisons between evertbrate nerves and the majority of unmyelinated vertebrate nerves will be of doubtful value, it having not been shown that similar Schwann cell-axon conditions prevail in the latter. My lipid results from lobster, clearly different from those in myelinated vertebrate nerves, in this connection tend to strengthen the suspicion of a species difference.

Alternative 2) will be further discussed below in connexion with alternative 4).

All authors seem to be agreed that the *fibrillar structures* in the axon chiefly consist of proteins. According to DE ROBERTIS and SCHMITT's very recent papers (41, 42) the fibrils are actually a kind of tubular formations. The wall of the tube, having a typical electrone microscopical pattern, predominantly contains proteins; any constituent lipid would be very strongly linked to the protein. The tubes are thought to contain a very dilute solution; little if any substance seems to occur inter-tubularly. This new structural picture of the axon — if it is confirmed — leaves little room for any lipids inside the axon. If so they would make up a very thin interneurotubular layer and/or surround the entire bundle of neurotubuli. In fact, DE ROBERTIS and SCHMITT in their preparations often find substances of lipid nature adhering to the neurotubuli but

assume them to be remnants of the myelin sheath. Their method involves fairly radical measures and perhaps entails the loss of easily destroyed or dissolved lipids; as yet their results are difficult to correlate with older conceptions. The fact remains, however, that the *essentially fibrillar* structures in the axon probably contain little substance of lipid nature, and alternative 3) may therefore be disregarded.

Many findings indicate the presence of lipids in the *interfibrillary substance*. In 1913 GÖTHLIN found that several types of axons were classifiable as myelotropic, others (among them axons in the retina of *Lepus cuniculus* and *Acanthus vulgaris* and partly spleen nerves of beef) as metatropic and this as a rule throughout the fibre, not only in the marginal portions. He states: »Übrigen dürfte es völlig gerecht fertig sein, *im allgemeinen* (italicized by the present author) in der interfibrillarsubstanz die Gegenwart von Lipoiden anzunehmen, and »Wenn bei den stabil proteotropen Nerven wirklich eine Spur von einer der Myelin der weissen Nerven homologen Substanz vorhanden ist, so ist sie zunächst in der Interfibrillarsubstanz zu suchen.« GÖTHLIN supposes that cephalin is the predominant lipid in the axon; it is suggested to have the most suitable solubility properties. In the intrinsically somewhat different fibres in the olfactory nerve of *Esox lucius* the lipid substance is apparently especially abundant and each separate fibril is characterized as almost being »einer ausserordentlich feinen markhaltigen Nervenrohre.« Cholesterol could be crystallized from this interfibrillary structure, which also was generative of myelin forms and stained like myelin.

The observations of ALSTERBERG, above described, bear out GÖTHLIN's; so do BEAR, SCHMITT and YOUNG's finding of a myelin form giving, P containing substance in truly sheath-free extrudates of squid giant nerve fibres. Consequently, alternative 4), perhaps partly in the form of alternative 2), seems to be most likely, at least as regards vertebrate axons generally. Then, the lipids I have attributed to the vertebrate axons would predominantly be interfibrillarily located — considering GÖTHLIN's results this is especially clear in respect of the retina axons. Such being the case, the phospholipids, according to ALSTERBERG's results, and perhaps



## General Summary

The aim of the work presented here was to reinvestigate the topical distribution of lipids in the nervous system by use of modern micromethods. Interest was especially directed towards the problem of the lipid contents in axons as opposed to that in the myelin sheaths.

I. For this purpose the requirements for and the applicability of lipid micromethods, based on the principle of characteristic constituent analysis, were examined (chapter I) and a lot of such methods, determining most known lipids present in nervous tissue, were tested (chapter II).

For reasons described some of the methods were selected for routine use. They include determination of total lipids (p. 38), lipid phosphorus, phosphorus releasable by mild alkali and acid treatment (p. 41), glycerol (p. 44), choline (p. 51), choline in lecithin (p. 59), ethanolamine (p. 62), amino acid (p. 63), hexose (p. 66), inositol (p. 48) and cholesterol (p. 64). Special attention was given to conditions at sampling, performance of lipid isolation from tissues by extraction and possible sources of error. Important observations were:

A. Chloroform-reextracts of alcohol-ether tissue extracts contain, in addition to all the lipids, some contaminants (pp. 32 and 36). These disturb significantly some of the methods employed: the determinations of inositol (p. 98), amino acids (p. 36) and total lipids; but the others little or not at all.

B. The presence in the nervous system of lipids, with phosphorus difficult to release by KOH acc. to SCHMIDT et al. (148) and other than sphingomyelin, is indicated (p. 42 and 161); so is the presence of phospholipids containing more than one mol. glycerol/atom P (p. 47 and 78)).

C. A diagram useful for the correction of the influence of cerebrosides in a sample on its glycerol value acc. to BLIX

(17) has been worked out (p. 46). Hydrolysis by  $\text{Ba}(\text{OH})_2$  in saturated water solution liberates all lipid choline (p. 57). A number of other technical details are discussed and improvements described. Possible means of further developing the methods are suggested.

D. A procedure (WEIL (189)) for lipid determination based on fractionation with solvents, included great errors. Their nature is penetrated in detail.

E. Autolysis of a duration and intensity, generally met with in autopsy materials, does not significantly influence the results on intact nervous tissue with the writer's methods (p. 83). Nervous tissue homogenates, especially when suspended in salt solutions, during autolysis lose some phospholipids, probably those containing sphingosine to the greatest extent. Other lipids are little or not at all affected.

F. During formalin preservation of nerve tissues phospholipids are lost, initially at a rapid rate, later more slowly but continuing (diagrams pp. 92, 93). The early loss consists of cephalins; a disappearance (breaking down) of the other phospholipids too becomes apparent only later. The study included materials preserved up to 9 years and the data obtained may be used as references in studies of pathological formalin-treated nerve materials.

Formalin treatment, even though short, renders part of the glycerophospholipids unhydrolyzable by mild KOH and acid treatment in the method acc. to SCHMIDT et al. (p. 94).

II. The experiences from the methodological study made it possible to perform an investigation of nerve lipid distribution similar to the one considered in the beginning of the summary (see also p. 100).

A. The chief lipids in the axon and nerve cell cytoplasm seem to be lecithin and phosphatidyl ethanolamine, almost equal in amount, viz. 1.1 % of fresh tissue and inter-fibrillarly (interneurotubularly) located. Gangliosides are probably also present, in a concentration of a few tenths of 1 %, and possibly cholesterol in minute amounts.

In the myelin sheath cholesterol and cerebroside predominate; the phospholipids present seem to be phosphatidyl serine, plasmalogens, sphingophospholipids and a little lecithin. The molar proportions between the lipid groups are approximately estimated as 8:4:2:2:2:1. As a group lipids make up some 35 % of fresh myelin sheath. Neutral fat and cholesteryl esters are generally absent.

B. The given lipid picture applies to normal conditions and was obtained in investigations of the following.

- 1) Normal mature grey and white matter from various sources, central and peripheral, of vertebrate and invertebrate nervous system (p. 118-122).
- 2) Developing nervous system of man and rat (p. 146).
- 3) Some pathological materials, viz. brain tumours (p. 150), spinal nerve during Wallerian degeneration (p. 162) and brain in demyelinating diseases (p. 167).
- 4) Also by correlation of chemical findings to known histological data and to results with recent histochemical and polarization optical methods (chapter XI).

In addition to the contributions to the chief result presented under II A, these studies gave many other experiences.

- a) Different brain cortex regions do not differ significantly as to the lipids analyzed (p. 109).
- b) »Grey» nerves contain lipids of a similar composition to those in cortex but in a lower concentration: the latter fact is assumed to be largely dependent on the dilution of the nerve elements by supportive tissue poor in lipids (p. 117).
- c) Lobster nerves differ from »unmyelinated» vertebrate nerves in showing a phospholipid picture composed about 80 % of a lecithin-like substance and containing practically no cerebroside (p. 107).
- d) White matter in spinal cord is definitely richer in lipids than brain white matter; the reason is thought to be a more abundant sheath supply in the former (p. 118).
- e) Even before stainable myelination has appeared, parts of the brain rich in axons have a greater lipid concentra-

tion than parts poor in axons. Thus, axons seem to contain more lipids (but of the same composition) than their parent cells (p. 146); at least before maturity.

f) Diagrams showing in detail lipid development in brain and spinal cord are presented (p. 140-143).

g) Even extreme deficiency in choline and thiamine during the early development of rats did not change the normal lipid formation; nor did A-avitaminosis, pantothenic acid deficiency of moderate degree or injections of gammexane (p. 132-133).

h) The fat deposits histologically identifiable by fat stains during the height of myelination are paralleled by some slight appearance of lipids of uncertain nature (possibly in part neutral fat p. 145).

i) No changes in the normal adult lipid picture are revealed by the author's methods as a cause of the visible accumulation of lipid pigment in advanced age (p. 139).

j) The lipid complex found in glioma and meningioma has a similar composition but is not as abundant as that in ordinary grey matter; it is supposed to be a general cytoplasmic constituent (p. 151).

k) The most striking event in respect to lipids during Wallerian degeneration was a large drop of sphingomyelins and a simultaneous parallel increase in choline-free KOH resistant phospholipid (fig. 5, p. 156). The latter is considered to consist of the rest of the sphingomyelin molecule remaining after choline liberation (p. 161). Lecithins are attacked late in the degeneration process; this is assumed to be dependent on a protected position relative to the other lipids (p. 160). After the leucocyte invasion cholesterol is esterified to a great extent. A rapid removal of all lipids then sets in (p. 162).

l) Diffuse sclerosis and encephalitis, diseases known to include demyelination, are accompanied by a loss, chiefly of those lipids in other experiments found to be typical of myelin sheath (p. 167-168).

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APPENDIX I  
TABLES

Table I. *Effect of Colloidal Iron Treatment on Extractibility of Brain Lipids*

	Grey matter				White matter			
	Cow I		Cow II		Cow I		Cow II	
	Direct analysis	Analysis after Fe treatment	Direct analysis	Analysis after Fe treatment	Direct analysis	Analysis after Fe treatment	Direct analysis	Analysis after Fe treatment
Dry substance	18.3	18.3	17.5	17.5	31.2	31.2	30.9	30.9
Total lipids	28.9	22.1	29.9	20.5	51.9	45.2	61.5	36.9
Phospholipids	17.6	10.4	17.1	10.9	22.1	15.1	26.5	11.3
Cholesterol	4.0	4.9	4.4	5.2	12.3	11.9	13.3	11.0
Cerebrosides	2.4	0	3.8	2.5	13.4	12.0	14.7	11.5
Unidentified	4.9	> 1.9 < 6.8	4.6	1.9	4.1	6.2	7.0	3.1
Lecithins	5.4	5.1	5.1	4.2	4.5	3.7	5.3	} 8.8
Cephalins A	9.6	3.9	9.9	5.1	11.8	8.1	15.2	
" B	0.5	1.1	} 2.1 (3.6)	0.8	2.2	2.1	2.5	} 2.5
Sphingomyelins	2.1	0.3		0.7	3.6	1.2	3.5	
" Diglycerides"	0.8	1.0	0.4	1.3	0.4	0	(-0.7)	(-0.2)
Choline phospholipids	7.5	5.4	8.7	4.9	8.1	4.9	8.8	5.2
KOH decomposable phospholipids	15.0	9.0	15.0	9.3	16.3	11.8	20.5	8.8
Glycerol as phospholipids	16.1	10.3	15.5	11.0	16.8	11.8	19.6	8.6

Table II. Analyses of Phospholipid Preparations

Contents of	Unit	Lecithin preparations			Cephalin preparations								Sphingomyelin preparations		
		A	B	C	A	B	C	D	E	F	G	H	A	B	C
					→ increasing alcohol and acetone solubility										
Phosphorus	%	2.95	2.91	3.74	14.5	3.47	3.37	3.35	3.25	3.38	3.37	2.52	1.45	2.83	3.54
KOH-releasable P	equ./equ. total P	—	0.04	0.93	1	0.92	0.96	0.83	0.80	0.77	0.79	0.75	0.55	0.24	0.16
Glycerol	"	1.00	0.64	0.93	—	0.94	1.04	0.93	1.30	1.17	1.05	1.27	0.53	0.27	—
KOH-releasable choline	"	0.54	—	0.85	—	—	0	—	—	—	0	—	0.23	0.14	0.09
Total choline	"	0.53	0.63	0.85	0	—	(0.04)*	—	—	—	(0.04)*(0.12)*	—	0.70	0.93	0.90
Ethanolamine **	"	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Serine**	"	—	—	—	—	+	+	—	—	—	—	(+)	—	—	—
Choline**	"	—	—	—	—	+	+	—	—	—	—	—	—	+	+
<i>Probable composition</i>															
Lecithins	% total phospholipids	54	64	85	0	92	0	83	80	77	0	0	23	14	9
Cephalins A	"	46	36	8	100	96	96	—	—	—	79	75	31	10	7
B	"	0	0	7	0	4	4	17	20	23	21	25	0	0	3
Sphingomyelins	"	0	0	0	0	8	0	—	—	—	0	0	47	79	81
"Diglyceride"	% relative to total phospholipid	0	0	0	0	2	4	10	50	40	26	51	0	3	—

Lecithin preparations: A and B: alcohol soluble fraction of ether fraction from grey and white matter, respectively.

C: lecithin from grey matter, highly purified according to Pangborn (134)

Cephalin preparations: A: highly purified brain inositol phospholipid, prepared acc. to Brante (27).

B, D and E: prepared acc. to Bliv (19a), being insoluble, slightly soluble and relatively well soluble in alcohol, respectively; F=fraction V acc. to Folch (65); H=supernatant acetone solution in the preparation of F; C and G: dialyzed phosphatidyl serine and colamine, respectively, prepared acc. to Folch (65).

Sphingomyelin preparations: A: ether insoluble substance in crude cephalin fraction of Folch; B and C: A, further purified by pyridine treatment and  $Al_2O_3$  adsorption procedures acc. to Klenk.

\* Colour of reprecipitate different (yellow) from that of choline reprecipitate.

\*\* By paper chromatography (600):  $\beta$  and  $\gamma$  occurring in  $\alpha$  not occurring.

Table III. Analyses of Lipid Fractions, Prepared from Brain According to Weil (189). (All Values are Expressed in % Dry Tissue).

Table III. Analyses of Lipid Fractions, Prepared from Brain Accidents. Expressed in % Dry Tissue).								
	Acetone I <sup>2</sup>	Acetone II <sup>2</sup>	Alcohol <sup>2</sup>	Ether	Pyridine	Chloroform-ethanol	Total contents obtained by Well's procedure	Contents in same material routinely analyzed
A. Grey matter								
Total lipids	13.6	10.8	4.3	2.7	1.6	1.1	34.1	37.9
Phospholipids	5.81	6.68	3.01	2.26	0.55	0.68	19.0	21.1
Cholesterol	5.60	—	0	0.04	0	0	5.6	5.9
Cerebrosides	0.86	2.27	1.13	—	1.11	0.49	5.9	6.9
Unidentified	1.3	1.9	0.1	0.4	0	0	3.7	4.0
Lecithins	2.89	2.93	0.05	0	0.09	0.09	6.1	6.1
Cephalins A	2.50	2.41	2.34	2.09	0.31	0.30	10.0	12.7
» B	0.29	1.34	0	0.09	0.05	0.07	1.8	0.1
Sphingomyelins	0.13	0	0.62	0.08	0.10	0.22	1.1	2.2
» Diglycerides»	0	0.40	(-0.23 <sup>1</sup> )	(-0.17 <sup>1</sup> )	(-0.04 <sup>1</sup> )	(-0.02 <sup>1</sup> )	(-0.06)	(-0.08)
Choline phospholipids	3.02	2.93	0.67	<0.08	0.19	0.31	7.2	8.3
» Diphosphoinositide»	—	0.21	<0.33	0.70	—	—	>1.2	2.2
By paper chromatography:								
ethanolamine	+	+	+	+	+	+		
serine	—	—	—	—	—	—		





Table IV. *Effect on Lipid Contents of Autolysis*

I. White matter		Cow I brain			Cow II brain		
		20 min. after death	Auto- lyzed 4 h. at 20°	Auto- lyzed 6 h. at 37° (subdi- vided)	30 min. after death	Auto- lyzed 2 h. at 25°	Auto- lyzed 6 h. at 37°
Dry substance	% wet tissue	32.3	32.3	32.4	32.5	32.0	33.2
Total lipids	% dry tissue	62.5	64.4	58.3	59.7	57.8	57.8
Phospholipids	»	28.6	28.0	25.4	27.0	26.5	26.0
Cholesterol	»	14.0	13.5	12.4	11.3	9.7	10.7
Cerebrosides	»	14.0	18.2	14.9	—	—	—
Unidentified	»	5.9	4.7	5.6	—	—	—
Lecithins	»	3.9	4.4	4.3	3.8	4.6	4.3
Cephalins A	»	18.0	15.2	13.9	15.8	14.1	13.8
» B	»	2.0	2.5	1.4	4.7	2.6	4.6
Sphingomyelins	»	4.7	5.9	5.8	2.7	5.2	3.3
» Diglycerides»	»	(—1.2)	0.8	0.7	0.1	0.4	1.1
Choline phospholipids	»	8.6	10.3	10.1	6.5	9.8	7.6
KOH decomposable phospholipids	»	21.9	19.7	18.2	19.6	18.7	18.1
Glycerol as phospholipids	»	20.3	20.7	19.1	19.8	19.2	19.5
II. Grey matter		cortex			cortex		
Dry substance	% wet tissue	17.9	17.4	19.7	17.4	17.3	—
Total lipids	% dry tissue	34.8	34.2	27.2	31.3	33.0	—
Phospholipids	»	21.6	22.1	15.8	20.1	20.5	—
Cholesterol	»	5.5	5.3	3.9	5.9	5.8	—
Cerebrosides	»	6.4	4.3	3.5	(5.2)	(1.7)	—
Unidentified	»	1.3	2.5	4.0	0	5.0	—
Lecithins	»	5.6	5.9	5.0	5.7	4.6	—
Cephalins A	»	11.4	11.5	9.8	10.7	12.2	—
» B	»	1.9	0.7	0.8	2.6	1.6	—
Sphingomyelins	»	2.7	4.0	0.2	1.1	2.1	—
» Diglycerides»	»	1.1	2.7	—	2.0	—	—
Choline phospholipids	»	8.3	9.9	5.2	6.8	6.7	—
KOH decomposable phospholipids	»	17.0	17.4	14.8	16.4	16.8	—
Glycerol as phospholipids	»	18.5	21.0	—	19.0	—	—

*Performed in Undiluted Tissue, Intact or Subdivided*

Cow III brain			Cow IV brain		Cow V brain		Cow VI spinal marrow		
2 h. after death	Auto-lyzed 24 h. at -18°	Auto-lyzed 24 h. at 25°	2 h. after death	Auto-lyzed 24 h. at 25° and 24 h. at 4°	1 h. after death	Auto-lyzed 48 h. at 25° and 24 h. at 4°	30 min. after death	Auto-lyzed 5 h. at 37° (sub-divided)	Auto-lyzed 15 h. at 37° (sub-divided)
32.1	31.6	30.5	31.6	31.8	33.8	32.9	36.8	38.5	44.0
62.3	61.7	58.7	67.1	60.1	60.9	63.2	70.9	78.4	81.8
25.6	27.0	26.0	28.5	25.3	27.9	27.1	31.1	31.8	32.8
12.9	11.3	12.7	15.4	13.5	11.2	11.8	15.7	17.2	19.0
17.5	16.2	15.9	18.3	—	17.3	9.2	19.5	21.7	19.5
6.3	7.2	4.1	4.9	—	4.5	15.1	4.6	7.7	10.5
}16.9	}19.7	}19.0	}20.1	}18.2	—	—	6.1	5.9	6.9
					}19.9	}19.0	13.9	14.8	15.3
}8.7	}7.3	}7.0	}8.4	}7.1			6.8	7.1	7.3
—	—	—	—	—	—	—	4.3	4.0	3.3
6.9	6.9	6.5	7.6	7.7	—	—	0.6	3.8	1.4
					8.0	8.1	10.4	9.9	10.2
16.9	19.7	19.0	20.1	18.2	—	—	20.0	20.7	22.2
—	—	—	—	—	20.8	—	20.8	25.7	24.0

n. caudatus			cortex		cortex		cortex (3 h)	
17.6	17.3	—	15.5	15.4	16.7	16.4	18.3	21.0
35.7	37.4	—	40.4	35.6	36.3	32.9	28.9	32.3
21.3	22.1	—	20.9	19.7	22.9	20.2	17.6	16.8
5.9	6.1	—	1.8?	5.3	3.8	4.3	4.0	4.2
5.6	6.3	—	9.6	2.2	7.1	5.2	4.9	—
2.9	2.9	—	8.1	8.4	2.5	3.2	2.4	—
18.3	19.2	—	19.4	18.4	—	—	5.4	5.1
3.0	2.9	—	1.5	1.3	13.1	11.9	9.6	11.0
—	—	—	—	—	—	—	0.5	0.7
8.4	8.7	—	8.0	7.8	9.8	8.3	2.1	—
18.3	19.2	—	19.4	18.4	—	—	0.4	—
—	—	—	—	—	—	—	7.5	6.1
							15.0	16.1
							15.5	—



Year	1950	1951	1952	1953	1954	1955	1956	1957	1958	1959	1960	1961	1962	1963	1964	1965	1966	1967	1968	1969	1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030																																																																																																				
Population	1,000,000	1,050,000	1,100,000	1,150,000	1,200,000	1,250,000	1,300,000	1,350,000	1,400,000	1,450,000	1,500,000	1,550,000	1,600,000	1,650,000	1,700,000	1,750,000	1,800,000	1,850,000	1,900,000	1,950,000	2,000,000	2,050,000	2,100,000	2,150,000	2,200,000	2,250,000	2,300,000	2,350,000	2,400,000	2,450,000	2,500,000	2,550,000	2,600,000	2,650,000	2,700,000	2,750,000	2,800,000	2,850,000	2,900,000	2,950,000	3,000,000	3,050,000	3,100,000	3,150,000	3,200,000	3,250,000	3,300,000	3,350,000	3,400,000	3,450,000	3,500,000	3,550,000	3,600,000	3,650,000	3,700,000	3,750,000	3,800,000	3,850,000	3,900,000	3,950,000	4,000,000	4,050,000	4,100,000	4,150,000	4,200,000	4,250,000	4,300,000	4,350,000	4,400,000	4,450,000	4,500,000	4,550,000	4,600,000	4,650,000	4,700,000	4,750,000	4,800,000	4,850,000	4,900,000	4,950,000	5,000,000	5,050,000	5,100,000	5,150,000	5,200,000	5,250,000	5,300,000	5,350,000	5,400,000	5,450,000	5,500,000	5,550,000	5,600,000	5,650,000	5,700,000	5,750,000	5,800,000	5,850,000	5,900,000	5,950,000	6,000,000	6,050,000	6,100,000	6,150,000	6,200,000	6,250,000	6,300,000	6,350,000	6,400,000	6,450,000	6,500,000	6,550,000	6,600,000	6,650,000	6,700,000	6,750,000	6,800,000	6,850,000	6,900,000	6,950,000	7,000,000	7,050,000	7,100,000	7,150,000	7,200,000	7,250,000	7,300,000	7,350,000	7,400,000	7,450,000	7,500,000	7,550,000	7,600,000	7,650,000	7,700,000	7,750,000	7,800,000	7,850,000	7,900,000	7,950,000	8,000,000	8,050,000	8,100,000	8,150,000	8,200,000	8,250,000	8,300,000	8,350,000	8,400,000	8,450,000	8,500,000	8,550,000	8,600,000	8,650,000	8,700,000	8,750,000	8,800,000	8,850,000	8,900,000	8,950,000	9,000,000	9,050,000	9,100,000	9,150,000	9,200,000	9,250,000	9,300,000	9,350,000	9,400,000	9,450,000	9,500,000	9,550,000	9,600,000	9,650,000	9,700,000	9,750,000	9,800,000	9,850,000	9,900,000	9,950,000	10,000,000

Table VI. *Lipids in Nervous Tissue Preserved in Formalin*

## I. White matter A. Human

	Before treatment	2 year old child I				2 year old child II		8 months old child III		Adult woman	
		After 36 days	After 87 days	After 125 days	After 200 days	After 2 years	After 2 years	After 3 years	After 3 years	After 8 years	After 9 years
					"grey part" "yellow part"						
Total lipids	55.2	53.9	57.2	54.7	56.3	61.9	50.4	40.9	49.2	15.5	
Phospholipids	26.2	22.8	22.9	20.3	21.9	21.7	17.8	12.7	10.0	3.5	
Cholesterol	10.8	13.1	12.8	12.6	13.5	14.7	11.6	9.5	10.8	3.6	
Cerebrosides	11.4	13.0	13.5	14.6	14.9	19.0	8.9	13.5	14.5	—	
Unidentified	6.8	5.0	8.0	7.2	6.0	6.5	12.1	5.3	13.9	—	
Lecithins	6.9		6.5	6.8	5.9	6.6	6.2	3.3	2.2	2.7	
Cephalins A	12.7	12.8	8.0	6.4	7.1	6.7	5.5	4.6	3.2	0	
"    B	4.2	10.0	5.6	5.0	7.6	6.7	3.4	3.2	2.5	0	
Sphingomyelins	2.4		2.8	2.2	1.3	1.7	2.7	1.6	2.1	1.0	
»Diglycerides»	3.2	5.8	—	3.0	4.5	3.7	2.1	1.2	1.4	?	
Choline phospholipids	9.3	9.8	9.3	9.0	7.2	8.3	8.9	4.9	4.3	3.7	
KOH decomposable phospholipids	19.6	12.8	14.5	13.2	13.0	13.3	11.7	7.9	5.4	2.2	
Glycerol as phospholipids	23.8	20.4	—	17.2	18.9	18.2	14.4	9.5	7.2	1.4	
»Diphosphoinositide«	1.7	0.5	—	—	0.7	0.7	—	—	—	—	

# B. Cow

	Before treatment				
	1/2 hour after death	4 1/2 hours after death (room temp., subdivided)	After 9 hours	After 7 days	After 60 days
Total lipids	61.5	62.3	69.1	58.0	56.3
Phospholipids	26.5	26.9	30.0	23.4	21.9
Cholesterol	14.0	14.1	17.3	15.4	14.0
Cerebrosides	14.7	15.9	19.7	16.1	16.8
Unidentified	6.3	5.4	2.1	3.1	3.6
Lecithins	5.3	5.7	6.6	5.4	(6.5)
Cephalins A	15.2	14.6	11.4	13.3	6.7
"    B	6.0	3.5	7.1		3.3
Sphingomyelins		3.1	4.9	4.7	5.1
» Diglycerides»	(- 0.7)	0.6	3.7	—	1.7
Choline phospholipids	—	8.8	11.5	10.1	11.9
KOH decomposable phospholipids	20.5	20.3	18.0	—	13.2
Glycerol as phospholipids	19.6	21.1	22.9	17.1	15.5

## Table VI (continued)

	2 years old child I			2 years old child II		Adult woman		5 months old fetus		Cow			
	Before treatment	After 36 days	After 125 days	After 2 years	After 8 years	After 9 years	Before treatment	After 12 hours	After 7 days	After 60 days			
Total lipids	37.5	30.4	35.7	26.2	22.5	17.6	29.9	34.6	35.9	36.7			
Phospholipids	22.0	18.3	17.6	12.8	4.5	2.8	17.1	21.9	21.3	20.1			
Cholesterol	5.7	6.1	6.1	5.8	5.0	4.6	4.4	5.0	6.6	7.3			
Cerebrosides	4.7	4.8	7.4	6.2	2.9	3.2	3.8	0?	3.9	6.3			
Unidentified	5.1	1.2	4.6	1.4	10.1	7.0	4.6	<7.7	4.1	3.0			
Lecithins	7.2	7.5	7.1	5.4	1.7	2.0	5.1	6.5	7.1	(9.3)			
Cephalins A	11.1	3.7	4.6	3.3	0.7	0.8	9.9	8.9	} 12.5	4.3			
"    B	1.7	4.5	3.2	2.4	0.9	} 0.0 (0.1)	} 2.1 (3.6)	3.7		5.0			
Sphingomyelins	2.0	2.6	2.7	1.7	1.2			2.8	1.7	1.5			
»Diglycerides»	1.4	4.8	3.6	1.3	1.6	1.9	0.4	—	—	1.7			
Choline phospholipids	9.2	10.1	9.8	7.1	2.9	2.1	8.7	9.3	8.8	10.8			
KOH decomposable phospholipids	18.3	11.2	11.7	8.7	2.4	2.8	15.0	15.4	—	13.6			
Glycerol as phospholipids	20.1	17.5	16.5	10.4	4.5	5.3	15.5	—	20.0	15.9			
»Diphosphoinositide»	—	—	1.6	—	—	—	—	—	—	—			

Table VII. *Lipids in Different Cortex Regions and Basal Gangliæ*

	12 years old boy							55 years old man
	C o r t e x				B a s a l   g a n g l i æ			
	Frontal type	Granular type	Agranular type	Polar type	Caput n. caudati	Thala- mus	Hypotha- lamus	
Dry substance	17.2	17.9	16.0	17.2	16.9	20.2	16.3	17.0
Total lipids	34.0	34.4	33.1	27.9	40.0	41.5	36.6	36.3
Phospholipids	19.5	19.7	20.2	17.0	22.5	21.7	20.2	18.8
Cholesterol	4.5	5.6	5.5	4.3	6.4	7.5	5.8	5.9
Cerebrosides	1.5	0 ?	1.9	3.1	2.7	7.0	4.8	3.7
Unidentified	8.5	9.1	5.5	3.5	8.4	5.3	5.8	7.9
Choline phospholipids	9.1	9.5	9.7	9.6	10.5	8.8	10.0	9.9
Cephalins	10.4	10.2	10.5	7.4	12.0	12.9	10.2	8.9



Table VIII *Lipids in Different*

## I. "Grey matter"

		Brain cortex			
		Human (12)*	Cow (5)*	Rabbit (6)*	Rat (2)*
Dry substance	% wet weight	15.1 $\pm$ 1.6	17.2 $\pm$ 1.1	18.9 $\pm$ 0.5	20.4
Total lipids	% dry weight	35.1 $\pm$ 5.0	34.3 $\pm$ 4.5	35.1 $\pm$ 0.9	34.6
Phospholipids	"	20.4 $\pm$ 1.2	20.6 $\pm$ 2.0	19.9 $\pm$ 0.3	21.9
Cholesterol	"	5.1 $\pm$ 0.7	4.8 $\pm$ 1.1	4.7	4.6
Cerebrosides	"	4.2 $\pm$ 1.9	7.0 $\pm$ 1.9	5.8 $\pm$ 0.6	6.1
Unidentified	"	5.4 $\pm$ 2.5	1.9	4.8	2.0
Lecithins	"	7.3 $\pm$ 1.3	5.6 $\pm$ 0.2	7.0 $\pm$ 0.3	7.5
Cephalins A	"	9.3 ( $\pm$ 1.8)	11.1	9.8 $\pm$ 0.8	11.9
" B	"	2.0 ( $\pm$ 1.1)	1.5	1.2 $\pm$ 0.5	0.6
Sphingomyelins	"	1.8 $\pm$ 0.7	2.4	1.9 $\pm$ 0.4	1.9
"Diglycerides"	"	1.1	—	1.1	(-1.4)
Choline phospholipids	"	9.1 $\pm$ 1.4	8.0 $\pm$ 1.1	8.9 $\pm$ 0.4	9.4
KOH decomposable phospholipids	"	16.6 $\pm$ 2.3	16.7 $\pm$ 1.4	16.7 $\pm$ 0.6	19.4
Glycerol as phospholipids	"	18.0 $\pm$ 1.1	—	18.2 $\pm$ 0.8	17.6
"Diphosphoinositide"	"	1.3 (4)*	—	—	1.2(1)*

## II. "White matter"

		Hemisphere central white	
		Human (12)*	Cow (12)*
Dry substance	% wet weight	29.2 $\pm$ 2.1	31.6 $\pm$ 1.2
Total lipids	% dry weight	61.2 $\pm$ 3.6	63.4 $\pm$ 3.1
Phospholipids	"	26.5 $\pm$ 1.6	27.4 $\pm$ 2.0
Cholesterol	"	13.8 $\pm$ 1.3	13.4 $\pm$ 1.5
Cerebrosides	"	16.0 $\pm$ 1.8	16.8 $\pm$ 2.2
Unidentified	"	4.9 $\pm$ 1.7	5.8 $\pm$ 1.9
Lecithins	"	5.7 $\pm$ 0.5	4.9 $\pm$ 0.9
Cephalins A	"	14.1 ( $\pm$ 0.6)	15.4 $\pm$ 1.7
" B	"	3.0 ( $\pm$ 1.2)	3.3 $\pm$ 1.8
Sphingomyelins	"	3.7 ( $\pm$ 1.3)	3.8 $\pm$ 1.0
"Diglycerides"	"	(-0.3)	0.8 $\pm$ 1.6
Choline phospholipids	"	9.4 $\pm$ 1.6	8.7 $\pm$ 1.6
KOH decomposable phospholipids	"	19.8 $\pm$ 1.3	20.3 $\pm$ 1.9
Glycerol as phospholipids	"	19.4 $\pm$ 1.8	21.3 $\pm$ 2.4
"Diphosphoinositide"	"	1.4 (5)*	—

\* number of separately analyzed materials on which the averages are based.

### Adult Nerve Tissues

Sympathetic gangliae	Internodular parts of sympathetic chain		Spleen nerves	N. vertebralis	Retina	Papilla n. optici	Ventral marrow	Claw nerve
Cow (1)*	Human (1)*	Cow (1)*	Cow (4)*	Horse (1)*	Cow (1)*	Cow (1)*	Lobster (1)*	
23.8	29.1	25.0	16.7	28.7	15.4	9.6	14.4	13.9
21.9	40.5	22.8	15.5	—	22.9	—	16.7	13.7
9.2	7.7	7.4	8.3	5.2	13.7	12.3	8.1	7.3
1.9	—	2.3	2.1	—	1.7	2.1	1.7	1.9
2.4	—	2.0	1.6	—	2.6	—	—	0.8
8.4	—	11.1	3.5	—	4.9	—	—	3.7
2.7	—	1.6	2.5	—	5.5	< 4.3	6.7	6.2
4.7	} 2.5	3.6	3.4	} 2.5	} 7.1	> 4.9	} 0.9	} 1.1
0.7		0.8	0.8			} 3.1		
1.1		—	1.4					
—	—	—	0.9	—	—	—	—	—
3.8	5.2	3.0	4.1	2.7	6.6	—	7.2	5.9
7.4	5.1	5.2	5.9	—	—	9.2	—	—
—	—	—	7.1	—	12.7	—	7.8	7.0
—	—	—	1.2(2)*	—	—	—	—	—

White matter from spinal cord				Intradural nerve roots		Nervus opticus	Periferal spinal nerves (ischadicus)			N. va- gus
Cow (3)*	Horse (1)*	Rabbtt (5)*	Cow (1)* ventral dorsal		Cow (2)*	Cow (1)*	Rabbit (7)*		Cow (1)*	
36.2	36.2	37.3 ± 1.9	29.0	33.8	33.0	38.0	35.4 ± 3.0	43.2		
75.8	74.6	69.4 ± 2.6	49.3	52.8	56.4	45.5	55.9 ± 3.7	35.6		
33.7	30.6	30.9 ± 0.8	25.3	27.7	22.8	16.1	27.4 ± 2.3	7.6		
15.9	—	15.9	12.2	12.6	12.0	6.2	11.5 ± 1.1	3.2		
19.7	—	17.2 ± 2.6	6.3	7.8	13.8	9.0	10.9 ± 2.4	2.3		
6.5	—	5.3	5.5	4.6	7.8	14.2	5.4 ± 2.3	22.5		
6.3	5.7	5.4 ± 0.2	4.9	5.7	3.4	2.1	3.6 ± 0.3	1.2		
17.1	16.2	16.0 ± 0.4	11.5	11.6	13.9	7.8	13.4 ± 1.3	3.9		
5.2	2.4	4.5 ± 0.9	2.6	4.1	1.1	2.3	4.1 ± 0.7	0.9		
5.1	6.4	5.2 ± 0.9	6.3	6.3	4.4	3.9	6.5 ± 1.8	1.6		
0	(-1.4)	2.6	0.2	0.6	(-0.3)	7.4	2.0	9.4		
11.4	12.1	10.6 ± 1.1	11.2	12.0	7.8	6.0	10.2 ± 1.7	2.8		
23.4	21.9	21.4 ± 0.5	16.4	17.3	17.3	9.9	16.9 ± 1.3	5.1		
23.4	20.1	24.8 ± 1.1	16.7	18.1	16.9	19.6	19.5	17.5		
2.1(1)*	—	2.1 (2)*	—	—	—	—	1.1 — 2.1(3)*	—		

Table IX. *Lipids in Human Brain at Different Stages of Development*

## I. Cortex

	Fetuses ("outer layer")							Children					
	3 (whole brain)	4 months old	4½ months old	5½ months old	7 months old	7½ months old	Pre- mature	Full term still- born	2 months	3 months	2 years	3 years	5 years
Dry substance	11.2	9.8	9.6	9.7	11.1	11.3	10.0	10.4	10.6	11.5	13.8	11.5	13.6
Total lipids	22.3	20.6	22.5	25.6	21.1	24.0	27.2	28.4	29.1	33.6	37.5	34.5	34.6
Phospholipids	13.4	12.7	12.4	13.8	13.7	14.1	16.7	16.8	17.0	21.6	22.0	19.7	20.1
Cholesterol	2.8	2.8	2.4	3.4	2.8	3.2	4.3	4.5	4.0	5.7	5.3	5.9	5.1
Cerebrosides	1.4	2.2	2.4	2.7	1.8	3.5	4.0	4.4	4.6	2.1	4.7	3.9	3.2
Unidentified	4.7	2.9	5.3	5.7	2.8	3.2	2.2	2.7	3.5	4.2	5.5	5.0	6.2
Lecithins	(2.2?)	>6.9	4.4	6.8	6.0	6.8	8.6	6.3	14.3	7.6	7.2	5.4	7.6
Cephalins A	11.9	5.4	7.1	6.4	6.7	6.5	5.4	9.8	9.8	8.7	11.1	10.6	9.3
B	1.5	0.4	0	0	1.0	0.8	2.7	0.7	2.7	3.9	1.7	0.8	1.8
Sphingomyelins	(3.9?)	(0.4)	0.9	0.6	(2.1)	(1.0)	2.7	(1.4)	2.7	1.4	2.0	2.9	1.4
Diglycerides	0.2	—	—	(-1.4)	0.2	(-0.8)	(-2.7?)	(-0.3)	2.0	1.9	1.1	0.8	1.3
Choline phospholipids	6.1	7.3	5.3	7.4	8.1	7.8	11.3	7.7	7.9	9.0	9.2	8.3	9.0
KOH decomposable phospholipids	11.9	12.3	9.4?	13.2	12.7	13.3	17.6?	16.1	14.3	16.3	18.3	16.0	16.9
Glycerol as phospholipids	12.1	—	—	11.4	13.0	12.2	14.1	15.7	16.9	18.8	19.8	17.0	18.6
"Diphosphoinositide"	—	—	—	3.6?	—	—	—	—	1.6	0.7	—	1.0	—

## Cortex (continued)

Age in years	10	11	12	16	19	55	65	72	75	76	78	79	90
Dry substance	17.3	15.9	17.2	14.5	15.1	18.0	14.7	13.5	14.8	13.5	13.2	13.8	12.3
Total lipids	—	31.8	34.0	27.8	37.9	27.2	40.0	42.1	36.3	40.6	32.5	36.0	38.2
Phospholipids	19.6	19.4	19.5	18.2	21.1	18.8	21.3	27.2	20.2	20.1	20.4	19.1	17.6
Cholesterol	4.7	4.1	4.5	5.3	5.9	3.9	5.9	6.2	5.2	5.8	4.8	5.2	5.6
Cerebrosides	2.0	1.6	1.5	4.1	6.9	—	5.1	3.4	5.3	6.1	4.1	6.1	5.3
Unidentified	—	6.7	8.5	0.2	4.0	—	7.7	5.3	5.6	8.6	3.2	5.6	9.7
Lecithins	6.6	16.4	12.1	5.9	6.1	12.7	7.4	8.4	6.8	8.3	10.0	6.6	6.6
Cephalins A	10.7	12.3	10.4	9.2	12.6	8.9	12.2	16.0	9.5	11.6	7.5	9.2	8.6
B	0.5	3.0	7.4	0.7	0	6.1	—	2.8	2.2	—	0.5	1.9	1.7
Sphingomyelins	1.8	—	—	2.4	2.2	—	1.7	(3.4)	1.7	0.2	2.4	1.4	0.7
» Diglycerides	0.2	—	—	1.1	(-0.3)	—	—	—	2.9	—	(-0.4)	1.1	2.3
Choline phospholipids	8.4	7.1	9.1	8.3	8.3	9.9	9.1	11.8	8.5	8.5	12.4	8.0	7.3
KOH decomposable phospholipids	17.3	16.4	12.1	15.1	18.7	12.7	—	24.4	16.3	—	17.5	15.8	15.2
Glycerol as phospholipids	17.6	—	—	16.6	18.3	—	19.0	—	20.1	17.9	17.0	17.2	18.2
» "Diphosphoinositide"	1.1	—	—	1.3	1.6	—	—	—	1.2	—	—	—	—

## Table IX. (Continued)

Fetuses										Children				
Age	"Inner layer"			"Inter-mediate layer"			"Inner layer"			brain	corpus callosum			
	4 months	4½ months	5½ months	7 months	7½ months	Pre-mature	Still-born	2 months	3 months			4 months	2 years	3 years
Dry substance	10.0	9.3	11.0	8.4	8.7	8.6	10.4	14.4	14.1	17.0	23.9	25.3	25.3	26.5
Total lipids	24.0	26.9	28.1	25.5	31.7	42.1	33.8	45.5	58.4	46.1	55.2	55.7	55.3	52.1
Phospholipids	14.0	16.7	16.5	15.8	19.2	22.9	19.1	22.7	28.3	24.6	26.2	25.6	24.1	22.6
Cholesterol free	3.2	3.9	4.4	4.0	5.2	5.9	5.4	7.2	9.7	8.7	10.8	12.2	12.4	11.2
Cholesterol total	3.1	4.0	—	4.2	5.2	6.5	6.3	8.9	9.3	11.6	11.4	13.8	13.0	12.2
Cerebrosides	4.7	—	—	2.6	2.1	2.8	3.8	10.4	9.3	11.6	11.4	13.8	13.0	12.2
Unidentified	2.1	—	—	2.9	5.2	9.9	4.6	3.5	11.1	1.2	6.8	4.1	5.8	6.1
Lecithins	7.1	7.2	6.9	8.1	6.4	10.2	8.9	7.8	8.9	8.5	6.9	7.0	5.6	5.2
Cephalins A	6.3	6.3	7.8	6.6	11.2	12.0	9.5	11.9	12.2	8.8	12.7	13.2	13.5	10.8
» B	0.4	2.9	0.3	0.1	1.6	0	0.7	1.4	6.0	6.2	4.2	3.3	2.5	4.6
Sphingomyelins	0.2	0.3	1.5	1.0	(3.3)	0.7	—	1.6	1.2	1.1	2.4	2.1	2.5	2.0
» Diglycerides	1.1	1.1	1.4	—	0.2	(—0.8)	0.1	1.9	2.7	0.4	2.9	1.0	0.2	1.1
Choline phospholipids	7.3	7.5	8.4	9.1	9.7	10.9	7.8?	9.4	10.1	9.6	9.3	9.1	8.1	7.2
KOH decomposable phospholipids	13.4	13.5	14.7	14.7	17.6	22.4	18.4	19.7	21.1	17.3	19.6	20.2	19.1	16.0
Glycerol as phospholipids	14.9	14.9	16.5	—	17.9	21.4	18.5	22.2	24.7	17.8	23.4	21.5	19.3	17.5
» "Diphosphoinositide"	—	—	—	1.6	3.4?	—	—	1.9	—	—	1.6	—	1.8	1.9

# Marrow (continued)

	Age in years	Children					Adults							
		10	11	12	16	19	48	55	55	55	65	72	75	78
Dry substance	% wet weight	31.5	29.6	29.9	32.0	29.8	30.3	28.4	27.3	31.2	28.3	27.5	24.5	26.5
Total lipids	% dry weight	60.3	64.9	62.5	55.3	60.4	61.7	64.8	61.9	67.0	61.5	58.2	55.5	62.6
Phospholipids	„	26.7	29.1	27.6	24.1	24.8	27.8	28.4	26.7	26.9	25.9	24.6	24.8	24.8
Cholesterol	„	13.7	13.4	15.1	11.9	15.1	13.5	14.1	13.1	15.6	13.6	14.3	12.3	13.1
Cerebrosides	„	14.2	14.1	15.2	15.0	15.0	17.7	16.6	18.9	17.5	18.2	16.1	13.3	15.4
Unidentified	„	5.7	8.3	4.6	4.3	5.5	2.7	5.7	3.2	7.0	3.8	3.2	5.1	9.4
Lecithins	„	6.0	21.9 21.1 7.2	—	5.7	4.7	5.6	—	—	6.2	6.0	6.0	18.6 12.6 6.2	5.5
Cephalins A	„	14.6		13.1	13.7	14.8	18.2	15.9	16.5	13.9	3.7	18.6		12.8
„ B	„	3.2		3.6	2.8	5.2								5.2
Sphingomyelins	„	2.9	—	—	1.7	3.6	2.2	—	—	4.2	2.3	—	—	1.3
„Diglycerides„	„	—	—	—	—	0.9	(- 0.8)	—	—	—	(-2.7)	—	(-1.1)	1.6
Choline phospholipids	„	8.9	8.0	11.0	7.4	8.3	7.8	10.2	10.8	10.4	8.3	—	12.2	6.8
KOH decomposable phospholipids	„	20.6	21.9	—	18.8	18.4	20.4	—	—	—	19.9	—	18.6	18.3
Glycerol as phospholipids	„	—	—	19.4	—	19.6	19.4	21.2	20.8	21.5	16.3	18.9	17.1	20.4
„Diphosphoinositide„	„	1.6	—	1.5	1.4	1.2	—	—	—	—	1.5	—	—	2.0

Table X. *Lipids in Rat Nervous System at Different Stages of Development*

I. Whole brain

	Age in days	C o n t r o l s				Choline deficiency		Thiamine deficiency		Pantothenic acid deficiency		Gammexane intoxication
		1	11	17	11	18	17	17	17	17	19	
Dry substance	% wet weight	12.4	13.8	17.4	14.3	17.3	17.6	16.2	18.1			
Total lipids	% dry weight	20.7	28.3	32.2	33.8	35.0	30.9	33.0	36.1			
Phospholipids	"	14.2	18.8	18.9	19.7	21.2	18.9	20.0	21.8			
Cholesterol free	"	2.9	4.7	3.9	4.8	4.7	4.9	3.7	5.2			
Cholesterol total	"	3.1	5.1	5.6	4.9	4.9	—	4.9	5.3			
Cerebrosides	"	2.2	3.0	3.7	3.1	3.5	3.5	3.3	4.3			
Unidentified	"	1.2	1.4	4.0	6.1	5.4	3.6	4.8	4.7			
Lecithins	"	13.4	15.5	16.2	8.3	18.5	8.4	8.0	18.2			
Cephalins A	"											
" B	"	0.8	3.3	2.7	0.0	2.7	2.1	2.3	3.6			
Sphingomyelins	"											
" Diglycerides	"	0.3	—	1.0	(-0.5)	0.9	0.9	0	0.5			
Choline phospholipids	"	7.3	9.7	8.5	9.7	8.8	—	8.5	—			
KOH decomposable phospholipids	"	13.4	15.5	16.2	18.3	18.5	16.8	17.2	18.2			
Glycerol as phospholipids	"	13.8	—	17.5	17.6	19.7	18.0	17.2	18.8			

## II. Cortex

	Controls					Choline deficiency				Thiamine deficiency				Pantothenic acid deficiency			Vitamin A deficiency		Gam-mexane intoxication
	Age in days	24	41	70		24	37	45		24	40	70		22	35		70	25	
Dry substance		18.7	20.0	20.4		20.0	20.2	20.5		19.2	21.3	20.4		18.9	19.9		20.2	18.2	
Total lipids	% wet weight	31.9	33.6	34.6		34.7	38.0	39.9		35.5	36.2	33.8		34.7	36.9		31.2	36.7	
Phospholipids	% dry weight	17.8	19.5	21.6		20.4	20.7	18.0		20.6	20.1	21.7		21.1	22.7		20.4	21.2	
free		3.7	4.4	4.6		4.3	5.0	4.0		4.4	4.5	4.7		4.7	5.1		4.7	4.8	
Cholesterol		3.7	5.1	4.7		4.5	5.0	4.0		4.0	5.5	4.9		4.7	5.1		4.4	4.9	
total		3.7	5.1	4.7		4.6	5.2	4.6		4.9	6.4	4.6		3.4	4.5		4.9	4.0	
Cerebrosides		3.9	5.0	6.1		4.6	5.2	4.3		5.6	4.2	2.6		5.5	4.6		1.2	6.6	
Unidentified		6.5	4.0	2.3		5.2	7.1	4.3											
Lecithins		6.8	7.0	7.5		18.4	6.9	6.6		18.3	7.4	8.1		8.5	7.2		6.6	17.2	
Cephalins A		9.0	9.9	11.9			9.8	8.0			11.5	11.0		10.8	10.3		10.9		
B		1.3	1.8	0.3			3.6	2.5		2.3		1.5		1.8	4.4		0.9	4.0	
Sphingomyelins		0.7	0.8	1.9		2.0	0.4	0.9			1.2	1.1			0.8		2.0		
Diglycerides		(-0.2)	0.2	(-1.4)		0.8	1.7	1.6		0.2	(-0.0)	(-0.6)		(-0.4)	1.8		0.3	1.4	
Choline phospholipids		7.5	7.8	9.4		—	7.3	7.5		10.5	8.6	9.2		12.8?	8.0		8.6	7.9	
KOH decomposable phospholipids		15.8	16.9	19.4		18.4	16.7	14.6		18.3	20.1?	19.1		19.3	17.5		17.5	17.2	
Glycerol as phospholipids		15.6	17.2	17.6		19.4	19.0	16.7		18.6	19.3	18.3		18.8	19.9		17.9	19.1	



Table X. (Continued)

## III. Spinal cord

		Controls						Thiamine deficiency				
		Age in days	1	11	17	24	41	70	17	24	40	70
Dry substance	% wet weight	15.9	15.9	18.1	24.1	25.2	27.9	32.0	25.2	26.4	30.6	33.1
Total lipids	% dry weight	24.2	24.2	36.7	44.0	51.6	56.6	57.4	40.0	49.6	58.8	52.9
Phospholipids	»	11.9	11.9	18.1	22.1	24.3	26.2	26.0	14.2	24.2	25.9	27.9
Cholesterol	free	3.2	3.2	5.7	5.2	8.4	10.9	12.1	3.7	8.1	10.9	11.8
Cerebrosides	total	—	—	—	5.3	8.6	10.9	—	5.4	8.1	10.9	—
Unidentified	»	—	—	—	5.6	10.5	16.4	14.3	4.6	11.1	16.7	6.1
					11.0	8.2	3.1	5.0	15.8	6.2	5.3	7.0
Lecithins	»	11.6	15.8	6.4	8.7	7.1	6.9		5.0	7.8	7.1	21.3
Cephalins A	»			10.7	11.5	12.0	13.0	9.2	12.6	12.3		
» B	»	0.3	2.3	3.6	3.6	5.9	3.0		0	2.2	5.8	6.6
Sphingomyelins	»			1.4	0.5	1.2	3.1	1.6		0.7		
» Diglycerides»	»	—	1.4	—	—	0.6	3.6	2.6	—	1.1	3.9	3.2
Choline phospholipids	»	7.8	7.1	7.8	9.2	8.3	10.0		5.3	9.4	7.8	7.5
KOH decomposable phospholipids	»	11.6	15.8	17.1	20.2	19.1	19.9		14.2	20.4	19.4	21.3
Glycerol as phospholipids	»	—	17.6	—	—	21.0	23.8	23.3	—	21.8	24.5	25.5

# Spinal cord (continued)

	Age in days	Choline deficiency				Pantothenic acid deficiency			Vitamin A deficiency		Gamma-xane intoxication	
		11	17	24	37	45	17	22	35	70	19	25
Dry substance	% wet weight	17.6	24.4	25.7	26.5	29.1	23.7	23.1	27.7	31.1	23.6	24.6
Total lipids	% dry weight	36.3	48.4	52.9	64.9	67.0	45.6	50.2	51.3	56.9	53.4	53.3
Phospholipids		16.6	23.9	26.4	28.0	27.3	21.4	24.8	25.4	27.8	25.0	26.5
Cholesterol free		4.5	6.0	7.7	11.5	12.1	4.1	7.8	—	13.5	7.9	9.2
Cholesterol total		—	3.5	9.0	11.7	12.0	—	7.5	9.3	—	8.1	9.4
Cerebrosides		—	7.8	11.6	13.7	17.0	7.3	9.3	10.1	15.7	12.2	10.8
Unidentified		—	10.2	5.9	11.5	10.6	12.8	8.2	6.5	(-0.1)	8.1	6.6
Lecithins		6.4	8.6	20.6	6.5	7.0	7.8	21.6	6.4	7.3	21.9	7.1
Cephalins A		8.9	9.9		13.8	11.7	9.3		11.5	14.0		15.8
"    B			5.4	5.8	6.7	7.0	4.0	3.2	7.0	4.8	3.1	2.6
Sphingomyelins		1.3			1.0	1.6	0.3		0.5	1.7		1.0
» Diglycerides		—	—	2.6	2.4	4.3	—	2.1	3.0	1.4	0	1.4
Choline phospholipids		7.7	—	9.1	7.5	8.6	8.1	—	6.9	9.0	—	8.1
KOH decomposable phospholipids		—	18.5	20.6	20.3	18.7	17.1	21.6	17.9	21.3	21.9	22.9
Glycerol as phospholipids		16.9	—	24.0	23.4	24.3	—	24.4	21.9	23.2	21.9	24.8

Table XI. *Lipids in Brain Tumours*

Table XI. Lipids in Brain Tumours

I. Mesodermal tumours

Mesodermal tumours															
	Meningeomas						Neurinomas			Haemangioma	Eosinophilic granuloma	Normal pia-arachnoides			
	1	2	3	4	5	6	1	2	3						
Dry substance	% wet weight	13.8	17.3	18.7	16.6	17.6	14.4	15.1	22.6	18.1	16.4	26.1			
Total lipids	% dry weight	14.1	18.0	25.1	16.3	20.2	--	11.1	18.2	20.3	25.6	23.4			
Phospholipids	"	8.4	10.5	10.6	7.7	8.8	8.3	8.7	6.9	7.5	6.1	5.8			
Cholesterol free	"	1.3	1.3	1.2	1.4	1.3	--	1.7	--	3.2	3.2	2.4			
Cholesterol total	"	1.4	1.8	1.8	1.9	--	--	2.2	--	--	8.4	6.3			
Cerebroside	"	(1.2)	3.2	3.0	0	--	--	--	--	--	--	--			
Unidentified	"	--	2.5	9.7	6.7	--	--	--	--	--	--	--			
Lecithins	"	--	8.2	8.2	5.8	3.4	--	--	--	3.3	5.4	>4.3			
Cephalins A	"	--	5.8	6.7	3.3	3.3	--	--	--	2.4	--	<0.4			
B	"	--	2.3	2.4	1.9	0.3	--	--	--	0.8	0.7	1.1			
Sphingomyelins	"	--	4.7	3.9	4.7	5.2	--	--	--	1.0	1.9	5.4			
Choline phospholipids	"	--	--	--	5.8	8.9	--	--	--	4.3	5.4	4.7			
KOH decomposable phospholipids	"	--	8.2	8.2	5.8	--	--	--	--	5.7	--	--			
Diphosphoinositide	"	--	--	--	--	--	<0.3	--	--	--	--	--			

## II. Ectodermal tumours

	Astrocytomas				Oligo-dendro-glioma		Ependy-moma		Spon-gioblas-toma		Medullo-blas-toma		Glioblastomas			Other malig-nant gliomas		Glio-matous of the tera-toma (forma-lin)		Glio-matous of the tera-toma (forma-lin)	
	I	II	III	IV									1	2	3	1	2				
Dry substance	13.2	16.2	15.1	11.6	16.0	15.7	13.6	17.4	16.1	14.0	17.6	15.8	10.3	17.3	15.7						
Total lipids	—	35.2	17.9	—	—	26.4	17.9	19.0	22.4	37.0	14.8	18.6	—	30.0	—						
Phospholipids	12.0	8.4	8.1	11.2	9.0	14.1	9.9	9.4	10.6	11.4	9.6	10.9	10.5	5.3	5.7						
free	—	—	1.3	—	—	1.3	1.7	1.6	1.6	—	2.2	—	3.2	1.6	—						
Cholesterol total	—	2.8	1.5	—	—	1.9	2.6	1.9	—	—	2.7	—	—	2.0	2.9						
Cerebrosides	—	—	—	—	—	2.4	4.4	—	2.9	—	—	—	—	2.0	—						
Unidentified	—	—	—	—	—	8.0	1.0	—	7.3	—	—	—	—	20.7	—						
Lecithins	10.1	7.7	7.0	7.1	—	10.6	2.4	4.4	8.5	9.6	—	4.5	—	2.7	4.0						
Cephalins A	—	—	—	—	—	—	5.7	5.0	—	—	—	—	—	1.3	—						
B	1.9	0.7	1.1	4.1	—	3.5	1.8	0	2.1	1.8	—	6.4	—	0.9	1.7						
Sphingomyelins	—	—	—	—	—	—	(2.9)	—	—	—	—	—	—	0.4	—						
Diglycerides	—	—	—	—	—	—	0.8	—	—	—	—	—	—	12.5	—						
Choline phospholipids	—	6.9	4.2	—	—	6.0	5.3	4.4	5.3	4.3	—	—	—	3.1	—						
KOH decomposable phospholipids	—	7.7	7.0	7.1	—	10.6	8.1	9.4	8.5	9.6	—	—	—	4.0	4.0						
Glycerol as phospholipids	—	—	—	—	—	—	9.2	—	—	—	—	—	—	16.5	—						
Diphosphoinositide	—	—	—	—	—	—	—	—	—	—	0.9	1.4	—	—	—						

Table XII. *Lipid Changes in Rabbit Ischiadicus During Wallerian Degeneration*

I. Unsevered control nerves

	Days after operation	1	3	6	8 a)	8 b)	13	16	19	23
Dry substance	% wet weight	36.7	35.9	39.4	39.9	37.3	32.9	31.8	38.5	32.5
Total lipids	% dry weight	54.2	44.6	52.8	—	—	56.2	52.5	56.1	62.5
Phospholipids	"	26.1	26.2	26.4	16.8	26.9	28.1	24.7	28.6	32.0
free	"	12.1	10.8	9.9	8.8	9.9	11.8	11.2	—	13.0
total	"	11.2	10.8	—	—	—	11.9	11.3	—	13.1
Cholesterol	"	9.5	9.5	8.6	—	—	12.7	10.1	3.0?	15.0
Cerebrosides	"	6.9	(—1.9)	7.9	—	—	3.5	6.4	—	2.4
Unidentified	"	(—0.6)	0	—	—	—	0.1	0.1	—	0.1
Cholesterolbound	"									
Fatty acids	"									
Lecithins	"	3.9	15.5	3.0	—	—	3.7	3.7	3.5	3.9
Cephalins A	"	12.5	15.9	13.5	—	—	14.1	11.4	13.5	15.3
B	"	5.1	10.7	4.0	—	—	3.5	4.1	4.8	3.1
Sphingomyelins	"	4.6		5.9	—	—	6.8	5.5	6.8	9.7
Diglycerides	"	—	—	—	—	—	0.8	2.1	1.1	2.9
Choline phospholipids	"	8.5	10.3	8.9	—	—	10.5	9.2	10.3	13.6
KOH decomposable phospholipids	"	16.4	15.5	16.4	—	—	17.8	15.1	17.0	19.2
Glycerol as phospholipids	"	—	—	—	—	—	18.8	17.8	18.5	23.0
"Diphosphoinositide"	"	1.1	1.0	—	—	—	—	2.1	—	—

## II. Degenerated nerves

Days after operation	‡	3	6	8 a)	8 b)	13	16	19	23
Dry substance	% wet weight	35.3	38.1	30.8	28.8	28.0	27.6	29.9	24.6
Total lipids	% dry weight	52.8	43.9	49.0	—	—	41.3	42.8	42.7
Phospholipids	»	25.3	22.4	23.7	15.5	23.9	15.5	15.2	13.6
free	»	11.3	10.0	8.3	8.5	9.5	7.9	—	6.0
Cholesterol total	»	11.4	10.3	8.5	—	—	10.1	—	11.9
Cerebrosides	»	9.6	7.7	7.5	—	—	9.6	3.4	6.5
Unidentified	»	6.5	3.5	9.3	—	—	6.1	—	10.7
Cholesterolbound									
fatty acids	»	0.1	0.3	0.2	—	—	1.5	—	4.1
Lecithins	»	4.0	}	3.1	—	—	3.7	3.2	2.9
Cephalins A	»	12.3		11.2	—	—	7.6	3.0	5.7
B	»	3.2		4.7	—	—	7.8	7.9	2.9
Sphingomyelins	»	5.8		4.7	—	—	1.7	0.9	3.4
» Diglycerides»	»	—	—	—	—	1.5	1.9	1.1	1.8
Choline phospholipids	»	9.8	7.4	7.8	—	—	4.6	6.6	8.4
KOH decomposable phospholipids	»	16.3	14.9	14.3	—	10.8	6.7	8.9	8.3
Glycerol as phospholipids	»	—	—	—	—	—	9.2	10.3	10.7
» "Diphosphoinositide" »	1.1	0.8	—	—	—	—	<1.2?	—	—

Table XIII. *Lipids in the Nervous System in Diffuse Sclerosis and Epidemic Encephalitis*

	Diffuse sclerosis						Encephalitis	
	Cortex			White matter			Cortex	White matter (Brain)
	I	III		Ia)	Ib)	III		
Case	13	9		13	13	9		
Age in months	1	11		1	1	11		
Length of formalin treatment in years								
Dry substance	—	—	% wet weight	—	—	—	14.1	29.6
Total lipids	23.2	25.9	% dry weight	14.9	17.2	23.4	41.9	46.3
Phospholipids	12.8	6.5		7.8	7.0	3.4	17.5	17.9
free	5.6	5.1		2.8	3.2	3.4	4.8	8.7
Cholesterol total	4.8	6.2		2.8	3.1	3.3	5.6	8.7
Cerebrosides	4.2	5.2		3.1	3.7	5.8	1.2	9.4
Unidentified	1.0	8.0		1.2	3.4	10.8	17.6	10.3
Lecithins	6.1	1.5?		3.3	3.2	1.3?		
Cephalins A	3.4	2.7		1.2		1.4	13.5	14.0
» B	3.3	1.0		3.3	3.0	0.2	8.6	10.6
Sphingomyelins	(3.6)	1.3		(3.9)	0.8	0.5	4.0	3.9
» Diglycerides»	0.8	(—0.5)		0.8	—	(—0.1)	(—0.2)	(—2.4)
Choline phospholipids	9.7	2.8?		7.2	4.0	1.8?	8.9	7.3
KOH decomposable phospholipids	9.5	4.2		4.5	—	2.7	13.5	14.0
Glycerol as phospholipids	10.6	3.5		5.6	6.0	2.6	13.2	10.8

## APPENDIX II

### METHODS



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## Subdivision of, dry substance determination in, and extraction of lipids from fresh tissues

### *Principle*

The tissues are rendered easy of access to the extraction liquids by being cut into sufficiently thin sections. A weighed part of the thus finely subdivided, mixed and weighed material is saved for extraction, the rest is dried and the decrease in weight through loss of water determined.

### *Quantitative application*

*Determination of dry substance.* For samples of 100—400 mg fresh tissue. (If the dry substance is required for determination of neuraminic acid at least 200 mg of grey matter or the like will be required.)

### *Extraction for lipid analysis.*

Complete analysis (see plan, p. 70): For 1700 mg grey matter or the like or 750 mg white matter.

Complete analysis without cephalin grouping: For 800 mg grey matter or the like or 350 mg white matter.

### *Reagents*

Alcohol-ether mixture, 3 parts 96 % alcohol to 1 of peroxide free ether.  
Chloroform, A. R. or redistilled, with 1 % ethanol added.

### *Accessories*

Freezing microtome

Stainless knife

Glass weighing jars with ground-on covers, 40 × 30 mm

Analytical balance

Vacuum exsiccator

Oven, for constant temperature of 104°

200 ml Jena or Pyrex round-bottom extraction flasks with 12 mm taper grinds

Reflux condenser with 12 mm taper grinds

250-500 ml Jena or Pyrex evaporation flasks with 26 mm taper grinds

Glass vacuum still with 26 mm taper grinds

Glass beads

Various measuring flasks (10—50 ml), standard quality

Various fat-free filter papers (Munktell 1 F boiled for 10 minutes each in two lots of alcohol-ether).

## Subdivision

Freeze dressed material on microtome table and cut into 7.5-10  $\mu$  sections and receive same on the edge of a stainless knife. A number of sections having been received scrape off knife on inside of a weighed weighing vessel, repeat procedure until enough material is available. The evaporation effect remains negligible since the knife is always scraped off on the same spot, thereby keeping the material cool. Close vessel tightly and weigh at once. Open vessel and stir rapidly with a long slender glass rod, partly transfer contents to an extraction flask, precharged with a suitable quantity (see below) of alcohol-ether (the material as a rule »drips« into the flask). Using the glass rod, quickly spread the material over the wall of the vessel and close same. Dip and move about in the alcohol-ether the end of glass rod, covered with material. Close extraction flask with a glass stopper and shake vigorously. Wash down any substance adhering to the wall of the flask with alcohol-ether. With a stainless knife scrape any material sticking to the glass rod into the flask which then is ready for extraction.

## Determination of dry substance

Weigh closed weighing vessel, open same and place in oven at 104° for 2 hours, cool in vacuum exsiccator over  $H_2SO_4$  for 30 minutes, close vessel and weigh. *N.B.* Before all the weighings wipe vessel with a dry towel, this includes the weighing of empty vessel, predried in oven and vacuum exsiccator.

### Computation

4 weights are obtained for each sample: 1-2 = weight of fresh sample to be extracted; 2-3 = weight of fresh sample for determination of dry substance; 3-4 = quantity of dry substance in 2-3. The water contents of the material can be computed from the 2nd. and 3rd. differences. The result of the determination of total lipids in fresh tissue (see below) can be used in finding the concentration of nonextractible substance.

If neuraminic acid is to be determined in the dry substance by Klenk's method (102), it is dried to constant weight in a vacuum exsiccator over  $H_2SO_4$  at room temperature.

## Extraction of lipids

### *Suitable quantities of extraction liquids*

For	Grey matter ml/g fresh	White matter ml/g fresh
alcohol-ether 3:1	about 100	about 150
chloroform	» 15	» 30—50

Extract sample well suspended in alcohol-ether by refluxion on water bath at 70° for 3-4 hours. Place flask overnight at room temperature and

filter contents completely through fat-free filter paper into a 250-500 ml evaporation flask with ground neck. Carefully save residue in extraction flask and later add to same a suitable quantity of chloroform.

Put some small glass beads or quartz lumps into the evaporation flask and connect same to a still provided with means for vacuum suction. Submerge flask slightly below inner liquid surface in a water bath at 60°. Agitating the flask, adjust by means of a pressure equalizing flask the vacuum suction to give moderate boiling in flask. Reduce temperature to below 50° at the end of the evaporation. Towards the end agitate the flask to prevent explosive bumping which often develops. When the substance seems perfectly dry submerge flask wholly below liquid surface for 1 minute. Remove top stopper and pour 5 ml of ethanol along the wall into the flask while tilting entire apparatus at various angles. Repeat evaporation as before at 50°. When the contents are dry increase suction to maximum for 1 minute with the flask completely submerged below the surface of the liquid. Again remove stopper and pour in enough chloroform down the wall. Disconnect flask from still and swirl around liquid for 5 minutes, heating at the end till liquid begins to simmer. Pour the hot liquid on same filter as before and filter into a suitably dimensioned measuring flask. Repeatedly wash evaporation flask with hot chloroform and via the extraction flask pour same on to filter. *Hereby the residue from the extraction is also subjected to extraction with hot chloroform.* Finally wash filter paper from the edge inwards with chloroform. At 20° fill the measuring flask to the mark. Shake measuring flask vigorously and as soon as possible apportion extract to the various procedures.

## Determination of total lipid contents

### *Principle*

Tissue lipids, selectively isolated by extraction, are freed from solvents, dried and weighed.

### *Quantitative application*

For 10 mg or more lipid extract.

### *Accessories*

Glass weighing vessels with ground on lids, 30 × 40 mm.

Electrical water bath of wood or asbestos with room for 10 weighing vessels.

### *Method*

Heat weighing vessels at 100° C. for a few minutes in a thermoregulated oven and cool under vacuum for 30 minutes. Thereafter weigh them, tightly closed and carefully wiped on the outside with a dry towel. Pipette the chloroform samples into such preweighed vessels; evaporate in a carbon

dioxide current on a water bath at 65-70°. Heat closed vessels for 2 minutes at 100°, vacuum exsiccate for 30 minutes, dry and weigh (the substance remaining in the vessels may later be used for determination of total choline).

### *Computation*

The difference between the former and the latter weights is used as a measure of the *total lipid contents*. If the figure thus obtained is subtracted from the quantity of dry substance in the tissue the result will be the quantity of substances inextractible with lipid solvents.

## **Determination of the total phosphorus contents in lipids**

[(Modified from Teorell (179), in its turn a modification of Fiske-Subbarow (62)]

### *Principle*

Inorganic phosphate, obtained by oxidation of lipid compounds containing phosphorus, in sulphuric acid solution is permitted to combine with ammonium molybdate to form phosphomolybdic acid. This is reducible with aminonaphtholsulphonic acid giving a blue colour which is colorimetrically determined by comparison to a standard with a wavelength of 720 mμ.

### *Quantitative application*

For samples containing 0.02-0.06 mg P, i.e. about 0.5-1.5 mg phospholipids.

### *Reagents*

Distilled water, free from phosphorus

Concentrated sulphuric acid, A. R.

30 % hydrogen peroxide, free from phosphorus (e.g. Perhydrol Merck)

Ammonium molybdate, A. R., in 5 % solution

1-4-6- aminonaphtholsulphonic acid (ANS)

Sodium bisulphite, A. R.

Sodium sulphite, A. R.

### *Accessories*

Pyrex tubes, 21 × 2 cm. Volume about 20 ml.

Sand bath with thermoregulator having a range up to 250°.

Pulfrich photometer or photoelectric colourimeter with 2 cm cuvettes and filter S 72.

### *Special preparations*

*Standard phosphorus solution* prepared from  $\text{KH}_2\text{PO}_4$  (crystalline, dried) and containing 0.1 mg P per ml.

*0.25 % 1-4-6- aminonaphtholsulphonic acid solution* prepared as follows: Dissolve 0.5 g ANS in 195 ml sodium bisulphite solution. Add 5 ml or

more of sodium sulphite solution until the liquid clears. Filter. Fresh ANS solution should be made up every third week at least.

15 per cent sodium bisulphite solution: Dissolve 30 g sodium bisulphite in 150 ml distilled water, Dilute to 195 ml.

25 per cent sodium sulphite solution: Dissolve 10 g  $\text{Na}_2\text{SO}_3 \cdot 7 \text{H}_2\text{O}$  in 20 ml distilled water.

#### *Purification of ANS for determination of phosphorus*

Put 60-70 g substance + 200 g pulverized borax + 450 ml distilled water in a porcelain dish. Filter mixture through a 10 cm Büchner funnel, wash with 100-150 ml distilled water. Add to filtrate a solution consisting of 850 ml 95 % alcohol + 150 ml HCl + a few drops bromine (agitate until all bromine dissolves), shake well and let stand for 5 minutes. Filter through 15 cm Büchner funnel. Wash with 700-800 ml 10 % NaCl solution. Repeat procedure with precipitate except that 300-400 ml 95 % alcohol + 200 ml ether is used in place of NaCl.

#### *Standardizing*

Concurrently with each test series 6 tubes, each containing 0.5 ml concentrated sulphuric acid only, are «oxidized» as below.

Then add to:

- 1) and 2) 22.5 ml distilled water
- 3) and 4) 2 ml standard phosphorus solution diluted 1 in 10 and 20.5 ml distilled water
- 5) and 6) 6 ml standard phosphorus solution diluted 1 in 10 and 16.5 ml distilled water.

Add thereafter to each of the 6 tubes 2 ml 5 % molybdate solution and agitate. Add ANS and read off in colorimeter as for lipid samples (see below). As blanks read tube 1) as the first in the series and tube 2) as the last.

#### *Determination in lipid samples*

Transfer an adequate quantity of lipid extract to a Pyrex tube. Evaporate solvent on a water bath, starting at 85° and increase gradually to 100°. Remove residual solvent (which would unnecessarily raise amount of  $\text{H}_2\text{SO}_4$  required for oxidation) by repeated blowing with  $\text{CO}_2$ . Wipe bottom of tubes, add to each 0.5 ml  $\text{H}_2\text{SO}_4$  and imbed them in mound of sand in a sandbath at about 250° for 3 hours. Shake tubes gently, remove them from sandbath and allow to cool for a moment and add to each 1 ml 30 %  $\text{H}_2\text{O}_2$ . Shake well so that contents washes walls of tubes and replace in sandbath for 1 hour or longer. Prevent tubes from spluttering—generally noticeable immediately after imbedding in sand—by careful observation during beginning of oxidation and removal to another place if tubes appear explosive where they are. If a tube should still be slightly brown 1 hour after the addition of  $\text{H}_2\text{O}_2$ , add 3 more drops of peroxide to all tubes and oxidize 1 hour more. Remove tubes and let them cool when all samples are completely oxidized and colourless. Shake tubes

## VIII

collectively against rubber bung after adding to each 22.5 ml distilled water and 2 ml 5 % ammonium molybdate solution. Note time and at 2 minute intervals add to each tube 1 ml ANS immediately followed by shaking and immersion in water bath at 25°. After exactly 20 minutes read off each tube in a colorimeter using 2 cm cuvettes and a filter for 720  $\mu$ .

### *Computation*

Plot a standard curve based on the extinction values for the standard samples and by introducing in the curve the individual extinction value of each sample its phosphorus contents may be obtained. Quantity of phospholipid = quantity of P  $\times$  25.

## Determination of phosphorus in easily splittable phospholipids ("KOH decomposable" phospholipids)

[(Modified after Schmidt et al. (148)]

### *Principle*

Unlike phosphorus in sphingomyelin the same element in lecithin and cephalin becomes acid soluble by mild treatment with KOH and that in acetalphospholipids by subsequent acidification. Total phosphorus in the solution is determined.

### *Quantitative application*

For samples containing 0.02-0.06 mg lecithin + cephalin phosphorus, i.e. about 0.5-1.5 mg lecithin + cephalin.

### *Reagents and accessories*

Same as for total phosphorus determination plus:

Trichloroacetic acid, A.R. in 3 and 12 % solutions

6 N Hydrochloric acid, A.R.

1 N Potassium hydroxide, A.R. Preferably this reagent should be freshly prepared for each series from a saturated KOH stock solution.

Small test tubes (12  $\times$  1.5 cm)

Funnels (largest diameter 5 cm)

Quartz lumps (half pea size), purified for analysis by repeated boiling in concentrated H<sub>2</sub>SO<sub>4</sub>.

Filter papers, Munktell OOR, 7 cm  $\phi$ . Should first be carefully washed a few times in 3 % trichloroacetic acid.

### *Standardizing*

Standard solution containing 0.04 mg P per ml is prepared by diluting 20 ml 0.1 mg P per ml standard solution (see determination of total phosphorus) with distilled water to 50 ml at 20°.

In small test tubes take:

- |      |                                  |                        |
|------|----------------------------------|------------------------|
| I.   |                                  | 4.2 ml distilled water |
| II.  | Exactly 1 ml standard solution + | 3.2 " " "              |
| III. | " 3 " " " +                      | 1.2 " " "              |

To each tube add: 1.8 ml 1 N KOH, 0.3 ml 6 N HCl and 2.1 ml 12 per cent trichloroacetic acid. Shake.\* Filter each sample into 10 ml measuring vessel through filter washed in trichloroacetic acid. Wash test tube with first 1 ml and then 0.5 ml trichloroacetic acid. Pour washing acid into measuring vessel and fill same with trichloroacetic acid until lower surface of meniscus is in level with 10 ml mark. Transfer with precision pipette into Pyrex tubes:

- |              |           |               |            |
|--------------|-----------|---------------|------------|
| 1) 5 ml I =  | 0 mg P    | 4) 3 ml II =  | 0.012 mg P |
| 2) 3 ml I =  | 0 mg P    | 5) 5 ml III = | 0.06 mg P  |
| 3) 5 ml II = | 0.02 mg P | 6) 3 mg III = | 0.036 mg P |

Thereafter, from and including the evaporation, treat standard samples like lipid samples.

#### *Determination in lipid samples*

Evaporate chloroform samples to dryness at 65-70° in carbon dioxide current. Dryness having been attained (complete absence of chloroform odour), cool tubes to room temperature and add to each 1.8 ml 1 N KOH, shake well\*, stopper and put into oven at 37°, shaking well\* every hour until a maximally fine emulsion is obtained and then at longer intervals. Remove tubes from oven and add to each 0.3 ml 6 N HCl. Shake.\*

Add 2.1 ml trichloroacetic acid, shake\*, let stand for 2 hours, add 4.2 ml distilled water, shake\*, let stand 20 minutes.

Now filter samples one by one into 10 ml measuring flasks through filters washed in trichloroacetic acid. Rinse each tube with first 1 ml and then 0.5 ml trichloroacetic acid and filter into the respective measuring flasks. Fill up to 10 ml mark. Filtrate should now be clear. Withdraw with precision pipettes one 5 ml and one 3 ml sample and put into Pyrex tubes.

To each combustion tube add some acid-treated quartz lumps and 0.5 ml concentrated  $H_2SO_4$ . Evaporate samples in heating chamber at 120°. Towards the end raise temperature to 145—150° for some hours. Transfer tubes to sandbath and oxidize for 1 hour, add to each 1 ml  $H_2O_2$ , shake along sides of tubes, heat for 1 hour, add 3 drops  $H_2O_2$ , shake, heat for another hour, remove from sandbath and direct carbon dioxide current into hot tubes. Determine phosphorus in by now clear and colourless samples colorimetrically as in total phosphorus method.

#### *Computation*

Cf. total phosphorus method.  $P \times 25 = \text{KOH decomposable phospholipids.}$

\* See that shaking is effective at bottom of tube.

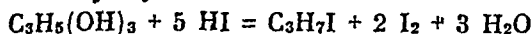


## Determination of glycerol in lipids

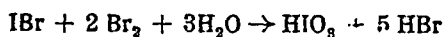
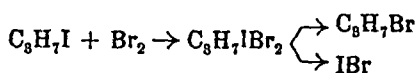
[According to Blix (17)]

### Principle

The glycerol is released from the lipids and converted into isopropyl iodide by treatment with hydriodic acid:



The isopropyl iodide is distilled in a special apparatus. On its way to the receiver the distillate is conducted through a thiosulphate solution to remove free iodine. The receiver contains a solution of bromine and sodium acetate in glacial acetic acid. Here the following reactions take place (in part first after the addition of water).



Excess bromine is removed by formic acid. The iodic acid is allowed to react with iodide at an acid pH and the iodine liberated is determined by thiosulphate titration.

### Quantitative application

For samples containing 0.2 mg or more of glycerol, i.e. about 1.7 mg or more of glycerol lipid.

### Reagents

Hydriodic acid, sp.gr. 1.70 for methoxyl determination acc. to Zeisel (Mfg. Ab Sv. Finkemikalier or Schering).

Acetic anhydride, A.R.

Red phosphorus

5 % sodium thiosulphate solution (aqueous  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ )

10 % sodium acetate (.3  $\text{H}_2\text{O}$ ) solution in glacial acetic acid

Bromine

Formic acid (85 %, Kebo)

4 % aqueous sodium acetate (.3  $\text{H}_2\text{O}$ ) solution

Dilute sulphuric acid (25 ml conc.  $\text{H}_2\text{SO}_4$  to 500 ml distilled water)

5 % potassium iodide solution

1 % soluble starch solution

0.02 N solution of sodium thiosulphate (factor set by potassium iodate titration, see Kolthoff (107)).

Glycerol, A.R. twice redistilled at  $161^\circ$  and 10 mm Hg pressure.

### Accessories

Modified Pregl apparatus according to Blix 1937 (17) with a narrow conical centrifuge tube as receiver.

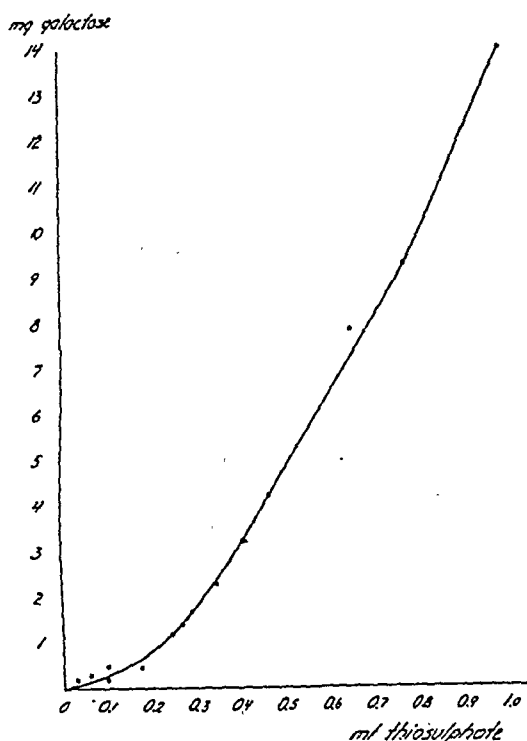
Nitrogen cylinder with reduction valve and washing flask containing 10 % NaOH

### Practical note

Hydriodic acid is very unstable and is best kept in small quantities in a brown bottle with a carefully ground-in stopper which after each opening

Figure 8

*Diagram for correction of glycerol values with respect to galactose in a sample.*



immediately is paraffined whereafter the bottle is put in a cool dark place. A carefully cleaned and dried pipette is necessary in removing contents from bottle (cf. Pregl (140)).

#### *Preparation of samples*

Evaporate to dryness a suitable quantity of chloroform extract in a microdistillation flask at 65-70° and with suction above the surface. Heat with suction for 20 min. at 80°. Leave overnight in evacuated exsiccator containing  $\text{CaCl}_2$  and paraffin on the bottom.

#### *Determination*

Assemble microdistillation apparatus. Lubricate stopcock and stoppers with a little distilled water before firmly and air-tight putting them in place. Avoiding formation of air bubbles, introduce about 0.3 ml 5 %  $\text{Na}_2\text{S}_2\text{O}_3$  into washing receiver. The thiosulphate must be a few mm above the lower end of the inner tube. Charge receiver with 3 ml 10 % sodium acetate in glacial acetic acid and 2 drops bromine and place below distillation tube so that it dips into solution. Now charge distillation flask with 0.2 ml acetic anhydride which dissolves substance during heating on

glycerol bath (approx.  $100^{\circ}$ ) with subsequent cooling to room temperature. Add a piece of red phosphorus the size of a pin point and lastly 2 ml hydriodic acid. Immediately afterwards fit distillation flask tightly to still, connect tubing from nitrogen cylinder to feeding tube and place whole assembly so that distillation flask dips  $\frac{1}{2}$  cm into glycerol bath with temperature  $120-125^{\circ}$ . Raise receiver so that internal tube almost touches bottom. Admit nitrogen at a rate sufficient to permit one bubble to leave bottom of receiver when preceding bubble has reached liquid surface. Arrange protective screen around flask and thermometer on top of bath and asbestos sheet between bath and receiver. Fasten some wet cotton wool around the widening on the distillation tube above the flask. Permit a small drops of distilled water to fall on the upper rim of the distillation flask to ensure that the upper half of the stopper is tight. Now the distillation process should continue for  $3\frac{1}{2}$  hours with control of temperature, gas flow and perhaps replacement of cotton wool, when necessary.

When distillation is finished lower receiver so that feed tube comes above liquid surface. Squirt outside of tube with some distilled water which may drip into receiver. Transfer quantitatively contents of receiver to a 300 ml flask and wash repeatedly by filling with distilled water and transfer this to 300 ml flask. Finally, having removed the stopper, wash inside also of feed tube with distilled water and let it flow into 300 ml flask.

Add 2 drops of formic acid to contents of 300 ml flask whereupon, shaking lightly, remove bromine by suction from water pump above surface. The liquid being absolutely colourless, add 25 ml 4 % sodium acetate solution and 5 ml sulphuric acid and shake. Add 2 ml 5 % potassium iodide solution and shake for a moment. Let stand for 1 minute. Then titrate with 0.02 N sodium thiosulphate solution until only slight yellowness remains. Add 1 ml 1 % starch solution and continue titration until lasting blue colour disappears for 1 drop sodium thiosulphate.

### *Blank*

0.2 ml acetic anhydride in a distillation flask is treated as above like a lipid sample.

A blank test is made for each new package of hydriodic acid and is repeated for control at intervals of 4 or 5 lipid samples.

### *Standardizing*

In the author's experiments a solution was used containing 0.5 mg glycerol per 0.2 ml acetic anhydride. The glycerol value for 0.2 ml of this solution was checked at least once for each new hydriodic acid package.

### *Computation*

No. of ml thiosulphate consumed by sample minus no. of ml consumed by blank gives quantity of thiosulphate in ml used up by the substance in the sample. The figure obtained is translated into mg glycerol by using the relation: 1 ml 0.02 N thiosulphate is equivalent to 0.307 mg glycerol. The calculated quantity of glycerol in the sample must be corrected by a

factor from the standardization of thiosulphate and if cerebrosides are present for their galactose contents (see below).

The thus corrected figure includes both phospholipid and neutral fat glycerol, if any. If the sample contains no neutral fat the figure can be directly converted to glycerol phospholipid by multiplication with 8.42. If the sample does contain neutral fat the phospholipid portion of the glycerol must be computed with the aid of the value for KOH releasable phosphorus and subtracted from the total quantity of glycerol. The difference times 9.62 is a measure of the quantity of neutral fat.

#### *Correction for cerebrosides.*

The amount of cerebrosides in the sample for glycerol determination is calculated. Its apparent glycerol value is taken from the diagram, fig 8, and subtracted from the glycerol value obtained by determination.

## Determination of total choline

[Modified from Entenman et al. (53, 54)]

### *Principle*

Choline is liberated from lecithin and sphingomyelin by means of hydrolysis with a suitable hydrolysis agent. After neutralization and filtering the choline is precipitated as reineckate the red colour of which is colorimetrically determined in acetone solution.

### *Quantitative application*

For samples containing 0.15-0.6 mg choline, i.e. about 1-4 mg choline phospholipid.

### *Reagents*

96 % alcohol

Barium hydroxide, A.R.

6 N hydrochloric acid

0.1 N hydrochloric acid

1.2 N hydrochloric acid

Reinecke salt (Hoffman-La Roche, purified as below)

Acetone, redistilled

Choline hydrochloride (Hoffman-La Roche, twice recrystallized from alcohol and dried).

### *Accessories*

Weighing vessels and water bath as for total lipid determination

Watch glasses suitable as covers for the vessels

Funnels, max. diam. 3 cm.

Filter papers, diam. 5.5 cm, Munktell IF.

Centrifuge tubes, conical, for Corda centrifuges with 16 tube holders (cf. cholesterol method).

Pointed glass rods, 3.5 and 12 cm in length.

Test tubes,  $10 \times 1.4$  cm

4 ml measuring flask

Photoelectric colorimeter or Pulfrich photometer with 5.0 cm cuvettes holding 1.1 ml.

### *Special preparations*

*Preparation of saturated barium hydroxide solution* (according to Entenman et al. (53)): Heat 200 ml distilled water to boiling for some minutes, add 20 g barium hydroxide while hot. Agitate for a short time. Filter with the aid of suction solution through large glass filter into suction flask. Decant from suction flask into storage bottle with rubber stopper. Allow to cool and crystallize before use.

*Purification of Reinecke salt (92)*: Pour 150 ml distilled water at  $50^{\circ}$  over 20 g Reinecke salt in a beaker. After stirring and when most of the salt has dissolved filter the solution at the same temperature (heated funnel). Put filtrate contained in a 100 ml centrifuge tube in refrigerator at  $-4^{\circ}$  for crystallization. Centrifuge after crystallization.

Pour away supernatant, dissolve and treat residue exactly as above but take only 100 ml distilled water.

Dry final residue between filter papers, shake it with minimum quantity of ice-cold 96 % alcohol, centrifuge off same. Finally pour on and centrifuge off minimum quantity of ether. Dry preparation in vacuum exsiccator.

*Preparation of reineckate solution in 1.2 N HCl*: Take about 200 mg purified Reinecke salt per 100 ml 1.2 N HCl, shake every now and then during 10 minutes, filter.

*Standard solution of recrystallized choline hydrochloride*: Prepare a stock solution by rapidly weighing exactly a quantity of dry choline hydrochloride and dissolving same in 1.2 N HCl. Check choline concentration in solution by N-determination according to Kjeldahl.

When needed prepare a 1:10 dilution of the stock solution with 1.2 N HCl.

### *Standardizing*

Simultaneously with each test series precipitate in the same manner a standard series made up from standard solution of 0.3 mg choline per ml 1.2 N HCl by instilling with a precision pipette into each tube:

- |    |     |    |          |         |          |      |    |     |   |     |
|----|-----|----|----------|---------|----------|------|----|-----|---|-----|
| 1) | 2   | ml | standard | choline | solution | +0.5 | ml | 1.2 | N | HCl |
| 2) | 1   | ml | »        | »       | »        | +1.5 | ml | 1.2 | N | HCl |
| 3) | 0.5 | ml | »        | »       | »        | +2.0 | ml | 1.2 | N | HCl |

To all tubes add 1.5 ml reineckate in 1.2 ml HCl, etc. (see below).

### *Determination in lipid samples*

*Hydrolysis*. After weighing (see Determination of total lipids, p. V) introduce along the walls of each vessel 1 ml 96 % alcohol. Heat briefly on water bath so that if possible the substance dissolves. Then add 2 ml saturated barium hydroxide solution to all vessels and also put same in

a 50 ml Erlenmeyer flask. Add to the latter 1 drop phenolphthalein and titrate with 6 N HCl from a 1 ml measuring pipette until the red colour disappears for  $\frac{1}{2}$  drop. Note quantity of 6 N HCl required.

Place all weighing jars in the test series in steam from a water bath. While alcohol evaporates liquid often exhibits tendency to foam which may be prevented by now and then carefully setting liquid in motion. When tendency to foam has completely disappeared (naturally too much liquid must not evaporate), add, if required, a small quantity of distilled water and cover opening of vessel with a watch glass. The hydrolysis may now go on for 1 hour, care being taken that liquid does not dry out nor foam up the walls and that the water bath contains enough water. After 1 hour add 2 ml more of  $\text{Ba}(\text{OH})_2$  solution. Repeatedly shake vessels with care during hydrolysis, remove watch glasses after 2 hours. Now allow liquid to evaporate until the substance just begins to dry at the centre of the bottom of vessel. At this moment remove same. N. B. Carefully watch vessel during evaporation, for drying sometimes may be very rapid.

To each of the thus hydrolyzed samples pour down wall of vessel the quantity (as measured by litre according to the above) required to neutralize the  $\text{Ba}(\text{OH})_2$  plus 0.5 ml of HCl. With a separate small glass rod for each vessel pry loose substance from walls and bottom so that all of it contacts liquid. Heat momentarily on boiling water bath (only long enough to make liquid quite hot, no significant evaporation may take place). Stir again with glass rod in vessel, crushing all adherent substance. Allow covered jar with glass rod inside to stand overnight.

Then filter liquid through minimal filter paper (Munktell 5.5 cm), pre-moistened with water, into a small centrifuge tube. Rinse weighing vessel three times with 0.5 ml 1 N HCl, tilting it to ensure complete washing of internal surface, and pour off onto filter over same spot on edge of vessel. Finally squirt 0.3 ml 0.1 N HCl along upper edge of filter to wash same. When everything has filtered through remove filter and blow any remaining liquid into funnel.

*Precipitation.* When all samples thus have been filtered and proved clear (after thorough shaking) add to each 1.5 ml Reinecke salt in 1.2 N HCl. Shake well. Allow samples to stand overnight at room temperature.

*Centrifuging and washing of precipitations.* Centrifuge tubes at 3000 revs. for 8 minutes. Decant off supernatant by a careful uniform movement controlling by eye that no precipitate accompanies the liquid (recentrifuge when there is a tendency to do this!). Blot away last drop of liquid by means of a clean filter paper.

Now pour down walls of tube 0.5 ml 1.2 N HCl and by slanting tube make it contact all parts of lower tube walls. Stir precipitate with a slender glass rod and remove same so that no precipitate goes along and centrifuge tube as before. Twice repeat washing with 1.2 N HCl and at the last washing the supernatant usually will be entirely colourless (if not wash all tubes once more).

After the last decantation add 1 ml of acetone along walls of tube, stir and centrifuge. Carefully decant solution into a small test tube without letting any deposit pass over. Wash in the same way two times more with acetone. *N.B.* After each centrifuging check against the light that the solution really is clear, also see that contents of the small test tube are absolutely clear. If such is not the case recentrifuge solution.

*Colorimetry.* When all samples in the test series are clear and dissolved in acetone, fill them up one at a time to a volume of 4 ml in a measuring flask, (Carefully wash tubes and funnel, each with at least three lots of 0.3 ml acetone.) Then colorimeter in the electrophotometer.

#### *Computation*

Plot a calibration curve with the aid of the extinction values of the standard series. The extinction values of the test series can be transformed into mg choline by means of the curve. The choline quantity  $\times 6.4$  = quantity of choline phospholipid.

### Determination of lecithin choline

[Modified after Hack (78)]

#### *Principle*

With the aid of mild treatment with KOH only that part of the choline which is bound to lecithin is liberated in acid soluble form. The hydrolysate is filtered after neutralization and its choline contents determined as described for total choline, p. XV.

#### *Quantitative application*

For samples containing 0.15-0.6 mg lecithin-choline, i.e. 1-4 mg lecithin.

#### *Reagents and accessories*

See total choline method

1 N KOH must be prepared afresh for each test series from saturated KOH solution

#### *Method*

Pipette chloroform extract into small pyrex test tubes. Evaporate chloroform in carbon dioxide current on a water bath at 65-70°. Add 1 ml 1N KOH to each tube. Put tubes into thermostatically controlled oven at 37° for 24 hours. Shake thoroughly every hour until maximum emulsification of lipids is attained. Then shake at longer intervals. Take out tubes and neutralize with HCl until a distinctly acid reaction is obtained. Keep tubes at 0° for some hours. Filter into centrifuge tubes through smallest possible filters. Add to each 0.5 ml 6 N HCl. Shake carefully, precipitate with Reinecke salt and determine as in total choline method (p. 000) using its standard curve for computation of the values.

#### *Computation*

See total choline method. Choline quantity  $\times 6.4$  = lecithin quantity.

## Determination of ethanolamine in lipids

[Modified after Blix (18) and Edman and Åquist (49)]

### Principle

Ethanolamine in lipids is set free by hydrolysis and then selectively distilled off. Amino nitrogen is determined in the distillate and thereby indirectly the ethanolamine contents.

### Quantitative application

For samples containing  $> 0.02$  mg ethanolamine-N, i.e.  $> 1.1$  mg ethanolamine cephalin.

### Reagents

6 N HCl (distilled according to Peters and van Slyke (137))

Calcium oxide, A.R.

0.1 N HCl

0.1 N NaOH

Glacial acetic acid, A.R.

Sodium nitrite

Potassium permanganate

Sodium hydroxide

Ethanolamine (purified as described)

} For determination of  $\text{NH}_2\text{-N}$ .

### Accessories

Edman's still (49) with ordinary small test tubes as receivers and a water bath as source of heat

van Slyke-Neill's manometrical apparatus

### Method

*Purification of ethanolamine* can be performed by redistillation 2-3 times of ordinary commercial ethanolamine in the Edman apparatus at  $80^\circ\text{C}$ . and 10-15 mm Hg pressure yielding an absolutely colourless product with a faint odour and with the nitrogen contents as  $\text{NH}_2$  agreeing with the calculated figure.

*Liberation of the base.* Evaporate in a 50 ml flask an aliquot (sufficient for serine determination also) of lipid extract. Add 4 ml 6 N HCl. Reflux mixture for 3 hours on a sand bath. Neutralize hydrolysate with concentrated NaOH solution (indication by dipping a thin glass rod in the solution and testing the pH on an indicator paper). Adjust pH to approximately 2-3 by adding 1 N HCl. Permit the mixture to stand for at least an hour for separation of the fatty acids. Then filter it through a Munktell OOR filter paper ( $\varnothing$  8 cm) into a suitable volumetric flask. Wash hydrolysis vessel at least three times with 1 or 0.5 ml portions of saturated NaCl solution and filter. Neutralize the filtrate to pH 7. Then fill the flask to the mark with saturated NaCl solution and shake. Lastly filter the contents into a test tube and put same, well-stoppered, into refrigerator until determination can be made (as soon as possible).



*Separation of ethanolamine by distillation.* Introduce a suitable quantity, (at most 3 ml) of the lipid hydrolysate into the distillation flask. Add 0.1 ml of 10 % CaO suspension. Drive off any  $\text{NH}_3$  present at  $40^\circ$  and highest vacuum possible. Then put in a receiver containing 0.5 ml 0.1 N HCl into which the tube from the condenser just dips (adjust this during distillation), and continue distillation at  $80^\circ$  and highest applicable vacuum, taking care that bumping over does not occur. Wind wet cotton wool around the stopcock to the cup to prevent overheating. When dryness is attained in the distilling flask reduce the pressure in the apparatus to 10-15 mm Hg and continue distillation for another 6 min. with flask completely submerged in water. Now wash down 1 ml distilled water from the cup into the flask and swirl it round the wall. Evaporate and repeat dry distillation for 6 min. as before. Remove receiver and neutralize with 0.1 ml 0.5 N NaOH.

*Determination of  $\text{NH}_2\text{-N}$ .* Wholly or partly transfer distillate into cup of van Slyke apparatus, in the former case washing out the tube a few times with distilled water. Then determination of  $\text{NH}_2$  nitrogen at 0.5 ml volume with no deviations from van Slyke's method (137).

#### *Computation*

Compute  $\text{NH}_2\text{-N}$  in mg according to Peters and van Slyke (137).  $\text{Mg N} \times 4.36 = \text{mg ethanolamine}$ . Quantity of ethanolamine  $\times 12.7 = \text{quantity of ethanolamine cephalin}$ .

## Determination of $\alpha$ -amino acid in lipids

[Acc. to van Slyke et al. (161, 83)]

#### *Principle*

The amino acids bound in lipids are liberated by acid hydrolysis.  $\text{CO}_2$  from the  $\alpha$ -amino-carboxyl group is driven out quantitatively by treatment with ninhydrin under suitable conditions. The quantity of  $\text{CO}_2$  is determined by gasometric methods and by means of the value obtained the quantity of  $\alpha$ -amino-carboxyl-N can be computed.

#### *Quantitative application*

For samples containing 0.03-0.05 mg  $\alpha$ -amino acid carboxyl-N, i.e. if the amino acid is serine 1.7-2.8 mg serine-cephalin.

#### *Reagents*

Ninhydrin, powdered, (LKB or Pfanstiehl)

Approx. 0.5 N NaOH solution as free as possible from  $\text{CO}_2$  in almost saturated NaCl solution

Approx. 5 N NaOH

Approx. 2 N lactic acid in almost saturated NaCl

Citrate buffer for pH 2.5 (acc. (161))

### Computation

Pressure of  $\text{CO}_2$  generated by sample =  $\text{PCO}_2 = P_1 - P_2 - c$ , where  $c = p_1 - p_2$  in blank.

$\alpha$ -amino acid carboxyl-N in mg may be calculated from  $\text{PCO}_2$  with tables by Mac Fadyen (116).

If the amino acid is assumed to consist solely of serine its quantity in the sample is determined by multiplying the  $N$ -value by 7.5. Quantity of serine  $\times 7.4$  = quantity of serine cephaline.

## Determination of inositol in lipids

[Modified after Beadle (12)]

### Principle

Free inositol in a lipid hydrolysate is allowed to supplement an inositol free culture medium for an inositol requiring organism. Within certain limits the maximum resultant growth is a measure of the inositol contents of the hydrolysate.

### Quantitative application

For samples containing 0.15-0.35 mg free inositol, i.e. 1-2 mg brain lipositol.

### Organism

*Neurospora inositolless* (No. 37401 obtained from G. W. Beadle, School of Biol. Sciences, Stanford University, Cal. U.S.A.).

### Reagents

Inositol free basal medium (see below for preparation).

Inositol, A.R.

Saturated NaCl solution.

### Accessories

Standard equipment for microbiological technique.

Pyrex round-bottom flasks with 12 mm taper grinds.

*Preparation of basal medium* (all substances A.R. quality).

	g
Ammonium tartrate	15
Ammonium nitrate	3
$\text{KH}_2\text{PO}_4$ (Sørensen)	3
$\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$	1.5
NaCl	0.5
$\text{CaCl}_2$ , anhydrous	0.3
Saccharose	60
Biotine	$15 \times 10^{-6}$

Include with each test series a standard series as per the above. From the results plot a standard curve with maximum quantity in mg of mycelium per flask and inositol quantity in  $\mu\text{g}$  per flask as abscissa and ordinate, respectively. If the curve consistently becomes rectilinear between 15 and 35  $\mu\text{g}$  the 20 and 30  $\mu\text{g}$  concentrations may be excluded from the standardisation.

#### *Determination in lipid hydrolysate.*

Hydrolysis (Woolley (197)). Evaporate a suitable quantity of chloroform extract in a 50 ml hydrolysis flask. Emulsify in distilled water while heating, add concentrated HCl until concentration in mixture becomes 20 %. Then reflux for 6 hours. Neutralize while shaking with concentrated NaOH, acidify to pH 2-3. Set aside for an hour or so. Filter sample into a measuring flask and repeatedly wash hydrolysis flask with saturated NaCl solution. Now neutralize to approximately pH 6 (indicating paper), fill up with saturated NaCl to desired volume and filter.

Pipette into each one of at least 9 flasks 1 ml of sample. Add 20 ml basal medium and subsequently treat sample like flasks in standard series. In so doing the growth in the test series must be estimated in relation to that in the standard series and the flasks should only be incubated for a period relative to their probable inositol contents, as a rule 5-6-7 days, 3 flasks taken daily.

If the sample is large enough the contents in two different dilutions of same sample should be determined.

#### *Computation*

The maximum mycelium due to the inositol in the lipid hydrolysate is calculated and plotted in the standard curve and the inositol contents read off.

## **Determination of free and total cholesterol in lipids**

[The Schoenheimer-Sperry method acc. to Sperry (1936)]

#### *Principle*

Cholesterol, preformedly free or released by hydrolysis, is quantitatively precipitated by means of digitonin. The purified precipitate is subjected to an empirically found and standardized colour reaction with acetic anhydride and sulphuric acid in acetic acid solution with subsequent colorimetry.

#### *Quantitative application*

For samples containing 0.02-0.06 mg free cholesterol or total cholesterol, respectively.

### Reagents

Mixture of redistilled acetone and ethanol, 1:1.

      "      "      "      "      "      peroxide free ether, 1:2

Approx. 0.4 % digitonin solution (prep. see below) (digitonin, Hoffman

La Roche or Merck)

Approx. 33 % KOH (preparation see below)

1 % alcoholic phenolphthalein solution

10 % acetic acid solution

Glacial acetic acid, A.R.

Acetic anhydride, A.R. (Merck)

Concentrated  $H_2SO_4$ , A.R.

Cholesterol, A.R. (if possible repeatedly recrystallized from alcohol)

1 mg/ml stock standard solutions of cholesterol, A.R., in glacial acetic acid and chloroform, respectively,

Working solutions of 0.1 mg cholesterol per ml in the above solvents

### Accessories

Slender, pointed glass rods approx. 13 cm long

1 l preserving jars with rubber gasket, half filled with sand

Pyrex or Jena centrifuge tubes with conical bottoms and holding approx.

12 ml, for Corda centrifuge with holder for 16 tubes

Dropping pipettes, 16 cm glass tubes with max. diameter 8 mm and provided with a drawn out, narrow, 6 mm long tip.

Rubber bulbs for the dropping pipettes.

Temperature regulated water bath for  $25 \pm 0.5^\circ$  provided with a rack for about 20 centrifuge tubes so arranged that they are in the dark and dip a few cm into the water.

Sand box with a sand layer about 3 cm thick.

Colorimeter (photoelectrical or Pulfrich photometer) with 1.1 ml micro-cuvettes of 5 cm length and filters with maximum absorption at 610 m $\mu$ .

### Preparations

**Digitonin solution.** Dissolve 400 mg digitonin in 100 ml distilled water. Let it stand for some days, a precipitate being formed. Now filter and repeat filtering immediately before use if the solution has become cloudy.

**KOH solution.** Take 20 ml of a concentrated solution (made from 51 g KOH and 49 g distilled water free from  $CO_2$ ) and dilute with 10 ml  $CO_2$  free water. Renew when cloudiness becomes marked.

### Standardizing

To test the reliability of the reagents and apparatus careful standardisation should be carried out before the method is started and also from time to time thereafter. Thereby will also be obtained the standard curve later used to convert the extraction samples into quantity of cholesterol. The level of this cholesterol curve is further checked by including in each series at least one standard sample (for example 0.1 mg cholesterol in 1 ml glacial acetic acid).

The more complete standardisation can advantageously be carried out with the following series:

Tubes 1) and 2)	1	ml working solution					
» 3)	» 4)	0.5	»	»	»	+ 0.5	ml solvent
» 5)	» 6)	0.2	»	»	»	+ 0.8	»
» 7)	» 8)					1.0	»

The solvent is either chloroform or glacial acetic acid. In the former case the entire determination procedure is carried through with and without hydrolysis and including digitonin precipitation. In the latter case only the colorimetric procedure itself is standardized. The curves should become rectilinear, pass through the origin and preferably coincide.

#### *Determination in lipid samples.*

*Free cholesterol.* Evaporate the samples (each containing at most 1 ml chloroform) to dryness on a water bath at 60-65°, blowing away the fumes with CO<sub>2</sub>. Add 2 ml acetone-alcohol to each sample. Heat while stirring momentarily the tubes in the water bath. After cooling add to each 1 ml newly filtered, clear digitonin solution and 1 drop of 10 % acetic acid. Stir vigorously until completely homogeneous. Stand samples with glass rods inside in tightly closed preserve jars overnight. After gentle stirring to loosen any particles of substance adhering to the tube at the surface of the liquid, remove rods without touching upper part of tube and place them on a wire frame in order so that no dried substance, if any, falls off. Centrifuge the tubes at 3000 revs. for 12 minutes. Carefully decant the supernatant liquid without disturbing the deposit and catch the last drop on a piece of filter paper. Add 2 ml acetone-ether, 1:2, stir, centrifuge for 10 minutes, decant, add 2 ml ether, stir, centrifuge for 8 minutes, decant. Repeat ether rinsing once.

Now place tubes in a shallow pan containing a 3 cm layer of sand which has been heated to 110-115°. Place in oven at same temperature for ½ hour. Remove sand bath and with tubes in the still hot sand accurately add 1 ml (standard pipette) of glacial acetic acid along wall of first tube and along lower part of glass rod in same. Shake vigorously. Leave the tube in the sand for about 2 minutes while adding acid to and shaking second and third tube. Then again stir first tube and remove it from the sand. Manipulate the remaining tubes as the first, then place all tubes with glass rods in them in a tightly closed preserve jar overnight.

Place tubes with glass rods inside for at least 5 minutes in a water bath protected from the light at 25°. For each series include a blank containing acetic acid only and a standard sample. At 5 minute intervals pipette in turn into each of the tubes 2 ml acetic anhydride and 0.1 ml sulphuric acid. Stir vigorously until completely homogeneous and return to the water bath. Keeping the temperature of the water bath constant at 25 ± 0.5°, remove tube no. 1 after exactly 30 minutes, take out stirring rod and draw up some of the solution in a pipette without disturbing any sediment, enough to rinse the cuvette twice. Decant carefully and

finally fill the couvette and read the extinction in a Pulfrich photometer. In so doing the blank couvette contains glacial acetic acid.

*Total cholesterol.* Evaporate samples as for free cholesterol but dissolve them in only 1 ml acetone-alcohol, add 1 drop KOH. Stir with a vigorous up and down motion of the rod until the drop of alkali at the tip of the rod disappears. Leave the rod in the tube. At the same time put all the tubes in a 3 cm layer of sand in a preserving jar where the temperature of the sand is  $45^{\circ}$ . Close the jar tightly and place for 30 minutes in an oven at  $37-40^{\circ}$ .

Remove the tubes, let them cool, take out the glass stirring rods, add 1 ml acetone-alcohol and 1 drop of phenolphthalein solution per tube, stir gently, titrate drop by drop with 10 % acetic acid (4-6 drops) until colour disappears. Add another drop of acetic acid and 1 drop of digitonin solution (clear) and henceforth treat the samples as for free cholesterol after the addition of digitonin.

#### *Computation*

By means of the standardisation curve the extinction values of the samples are converted into quantity of cholesterol.

## Determination of hexose in lipids

[Acc. to Brand and Sperry (23)]

#### *Principle*

The reductive power in an aqueous extract of evaporated lipid extract is determined before and after hydrolysis. The increased reductive power induced by the hydrolysis is due to hexoses chemically bound in the cerebrosides and with a knowledge of their type and normal quantitative occurrence in cerebrosides the latter may be quantitatively computed.

#### *Quantitative application*

For samples containing 0.1-0.6 mg hexoses, i.e. about 0.5-2.8 mg cerebroside.

#### *Reagents*

6 N HCl

Slightly more than 6 N NaOH (free from  $\text{CO}_2$ ).

Approx. 1 N HCl

0.1 N NaOH

0.1 % aqueous chlorophenol red (a few drops of 0.1 N NaOH added to dissolve indicator).

4.5 % zinc sulphate solution (prepared from  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ )

Alkaline ferricyanide solution (preparation, see below)

Approx. 18 N  $\text{H}_2\text{SO}_4$

0.15 N stock solution of cerium sulphate " " "

0.003 N working solution of cerium sulphate " " "

0.1 % solution of Setopaline C (J. R. Geigy, Basle, Switzerland) (preparation, see below)

Washed 7 cm filter papers (preparation, see below)

#### *Accessories*

Water bath with holders for 10 hydrolysis flasks at the same time.

Hydrolysis flasks, round bottom, of relatively thin Jena or Pyrex glass holding 10 ml and provided with long melttable neck (15 cm).

50 ml Erlenmeyer flasks of Jena or Pyrex glass

Standard microburette, total vol. 2-5 ml, graduated in 0.01 ml

#### *Special preparations.*

6 N HCl. There is used a distillate according to Peters and van Slyke (137).

6 N NaOH. 34.3 ml concentrated NaOH solution (50:50) is let out of a pipette below the surface of about 50 ml cold, boiled, distilled water in a 100 ml measuring flask. Shake while cooling in running water. Fill to the mark with boiled, distilled water at 20° C.

Approx. 18 N H<sub>2</sub>SO<sub>4</sub>. During water cooling add 213.9 g concentrated H<sub>2</sub>SO<sub>4</sub> to 133.8 g water. (The conc. sulphuric acid may not contain any reducing substance. Test as follows: To 20 ml acid add 60 ml water and 0.05 ml approx. 0.1 N potassium permanganate. The red colour should remain for at least 5 minutes.)

*Setopaline C solution.* Prepare as needed in suitable quantities by dissolving 1 mg Setopaline C per ml distilled water.

*Stock solution of cerium sulphate.* Mix and heat slightly 20 g cerium sulphate (Merck with 4 H<sub>2</sub>O), 7 ml concentrated H<sub>2</sub>SO<sub>4</sub> and 7 ml distilled water. Add small portions of water until everything is practically dissolved. Then filter solution and refill to 200 ml. To exactly 15 ml samples add 50 ml water and 3 ml 18 N H<sub>2</sub>SO<sub>4</sub> and titrate with 0.1 N Mohr's salt (39.214 g Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>·6H<sub>2</sub>O, A.R., and 25 ml 18 N H<sub>2</sub>SO<sub>4</sub> in 300 ml water, afterwards diluted to 1 l) until colour almost fades away. Add 8 ml Setopaline C and continue titration until a sharp colour change takes place from golden brown to straw yellow. Then the normality of the cerium sulphate solution will be = volume of Mohr's solution/volume of cerium-SO<sub>4</sub> solution × 0.1000. With the aid of the found value the solution can then be diluted with distilled water to exactly 0.15 N. Store in brown bottle.

*Dilute cerium sulphate solution.* Prepare as needed by diluting with distilled water exactly 2 ml stock solution and 5 ml 18 N H<sub>2</sub>SO<sub>4</sub> to exactly 100 ml.

*Alkaline ferricyanide solution.* Dissolve in water to 500 ml 2.5 g purified (cf. Folin (70)) K<sub>3</sub>Fe(CN)<sub>6</sub> and 5.3 g anhydrous Na<sub>2</sub>CO<sub>3</sub>. Store in brown bottle in the dark.

*Washing of filter paper.* Wash Munktell OOR 7 cm filter papers by boiling them twice for 5 minutes in distilled water. Rinse in distilled water and dry in air.

*Standardizing*

*Standard solution.* 0.3 ml galactose per ml.

*Standard series*

Flask 1) and 2)	2 ml	standard solution	= 0.6 mg galactose
3) and 4)	1 ml	»	= 0.3 mg »
5) and 6)	0.4 ml	»	= 0.12 mg »

Add to each odd sample 2 ml 6 N HCl. Then dilute all samples to 4 ml with distilled water. Boiling and other treatment as for the corresponding lipid samples, this also applies to blanks.

*Determination in lipid samples.*

*Hydrolysis.* Pipette equal quantities of a lipid extract into each of two hydrolysis flasks (one with an odd number and the other with the next highest even number) taking care that the whole sample lands in the middle of the bottom of the flask. After removal of the solvent by mild heating (60-70°) in carbon dioxide current or with suction above the surface, add to each flask 2 ml redistilled water. Place flasks in boiling water bath for 5-10 minutes, swirling contents from time to time to emulsify the lipids. Then add to all odd flasks 2 ml 6 N HCl and to all even ones 2 ml redistilled water. Swirl contents round inside flask.

As blanks pipette into an additional (odd flask) 2 ml 6 N HCl + 2 ml redistilled water, into another (even) 4 ml redistilled water.

Seal necks of flasks by melting over a flame and then heat in boiling water bath for 45 minutes. (Swirling after 15 and 30 minutes.)

*Neutralization.* After cooling add one drop of chlorophenol red and 2 ml 6 N HCl to all odd flasks. Then add 1 N HCl drop by drop until solution becomes acid (yellow) and lastly bring colour to a distinct purple by careful drop-by-drop addition of 0.1 N NaOH. To each even flasks add 1 drop chlorophenol red, 2 ml distilled water and 0.1 N NaOH until colour becomes distinctly purple.

*Clarification.* After cooling add 0.5 ml zinc sulphate solution to all flasks, followed by 2 ml 0.1 N NaOH. Shake samples vigorously then let them stand for a little while until deposit is formed, surrounded by clear solution.

*Filtering.* Transfer solution quantitatively through a washed filter to a 50 ml Erlenmeyer flask of Pyrex. Wash four times hydrolysis flask and filter with 3 ml portions of distilled water under such conditions that none of the original contents of the hydrolysis flasks gets lost. The filtrates should be clear or at most slightly opaque. Refilter through the same paper cloudy filtrates.

*Oxidation with ferricyanide.* Add 2 ml ferricyanide solution to each flask and then submerge them in a special holder into an actively boiling water bath for exactly 15 minutes. Take out flasks and let them stand in running water for 3 minutes. Then set them aside for subsequent titration which must be carried out within 1 hour.



*Titration.* Add to each sample about 1 ml 18 N  $\text{H}_2\text{SO}_4$  followed by 7 drops indicator immediately before titration of the sample. Titrate solution with dilute cerium sulphate solution in white light against a white background until the straw yellow colour turns golden brown. (N.B. The transition must be sharp and must not be confused with moderately deepening colour during the titration.) Set the burette to give a relatively rapid stream of drops. The colour should change for 1 drop.

### *Computation*

Assuming the consumption in ml of cerium sulphate to be:

for hydrolyzed sample  $x$ , unhydrolyzed d:o  $z$ ;

for hydrolyzed blank  $y$ , unhydrolyzed d:o  $q$ ;

and that 1 ml cerium sulphate is used up per  $k$  mg galactose, the lipid galactose contents of the sample will be expressed by the formula

$$[(x-y) - (z-q)] \times k$$

Quantity of galactose  $\times 4.55 =$  quantity of cerebrosides.

## Errata

p = page. l = line.

Change the following:

- p. 13, l. 7, and p. 62, l. 2. »monography» to »monograph».  
p. 14, l. 26. »16-26» to »16-24».  
p. 22, l. 5. »individable» to »indivisible».  
—, l. 35. »hexacosanoyl» to »hexacosenoyl».  
p. 27, l. 34. »exraction» to »extraction».  
p. 46, l. 1. »by» to »but».  
p. 47, l. 4. »be» to »been».  
p. 49, l. 36. »HC1» to »HCl».  
p. 62, l. 10. »CH1» to »HCl».  
p. 64, l. 30. »Doktor» to »Doctor».  
p. 67, l. 2 and l. 27. »orcine» to »orcinol».  
p. 67, l. 13. »differentation» to »differentiation».  
p. 68, l. 12. »opalscence» to »opalescence».  
—, l. 3. »karbazol» to »carbazole».  
p. 73, l. 31. »suitably» to »suitable».  
p. 100, l. 19. »Caspersson's» to »Caspersson».  
p. 104, l. 7, p. 105, l. 8, p. 108, l. 29. »symphatetic» to »sympathetic».  
p. 104, l. 8, p. 105, l. 15. »symphiticus» and »symphaticus» to »sympathicus».  
p. 109, l. 13. »reiable» to »reliable».  
p. 114, l. 9. »36 04» to »36.04».  
p. 116, l. 6. »11 6» to »11.6».  
—, l. 24. »characteristics in the species» to »(79) and will therefore not be discussed here».  
p. 124, l. 32. »33 day» to »33rd day».  
p. 126, table. »subduralae» to »subdurale».  
p. 127, l. 38. »19 2» to »19.2».  
—, l. 45. »symptons» to »symptoms».  
p. 128, l. 17. »symptions» to »symptoms».  
—, l. 22. »gammeexane» to »gammexane».  
p. 132, l. 33. »70 days group» to »in 70 days group».  
p. 136, l. 9. »considerable phospholipids» to »considerable amounts of phospholipids».  
p. 155, l. 1. »free» to »total».  
p. 157, l. 4. »absolute» to »absolutely».  
p. 159, l. 45. »hokever» to »however».  
p. 161, l. 10. »enzymes those» to »enzymes are those».  
—, l. 20. »reproducable» to »reproducible».  
p. 167, l. 23. »hitologically» to »histologically».  
p. 170, l. 15. »method» to »methods».  
p. 171, l. 10. »olycolipids» to »glycolipids».  
—, l. 11. »thionin» to »thionine».  
p. 173, l. 10. »precitation» to »precipitation» and »JCN» to »ICN».  
—, l. 28. »protaplasm» to »protoplasm».  
p. 177, l. 30. »electrone» to »electron».  
p. 178, l. 11. »Acanthus» to »Acanthias».  
p. 180, l. 14. »relaasable» to »releasable».  
Appendix I, table II, last line »(000)» to »(24)».



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CENTRAL AND CHEMOREFLEX  
COMPONENTS IN THE  
RESPIRATORY ACTIVITY DURING  
ACID-BASE DISPLACEMENTS  
IN THE BLOOD

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*With an Appendix*

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*By*  
CARL MAGNUS HESSER

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STOCKHOLM

1949



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## APPENDIX

- A Device for Measuring and Recording Automatically and Continuously the Carbon Dioxide in the Alveolar Air.

## I. Preface.

The investigations to be presently described were carried out during the years 1947—49. The work is to a great extent a continuation of earlier activities in this laboratory pertaining to the field of respiration physiology.

It is a great pleasure for me to express my deepest gratitude to the Director of the Physiological Laboratory, Professor ULF VON EULER, for most valuable advice and stimulating encouragement during these years.

I also wish to convey my heartfelt thanks to Docent HILMING BJURSTEDT of the Department of Aviation and Naval Medicine, for kindly placing at my disposal part of the technical set-up in the laboratory, and for supporting my interest in this work.

To ARNE ÅSTRÖM, Research Assistant, I am greatly indebted for obliging and never-failing help in the experimental procedures.

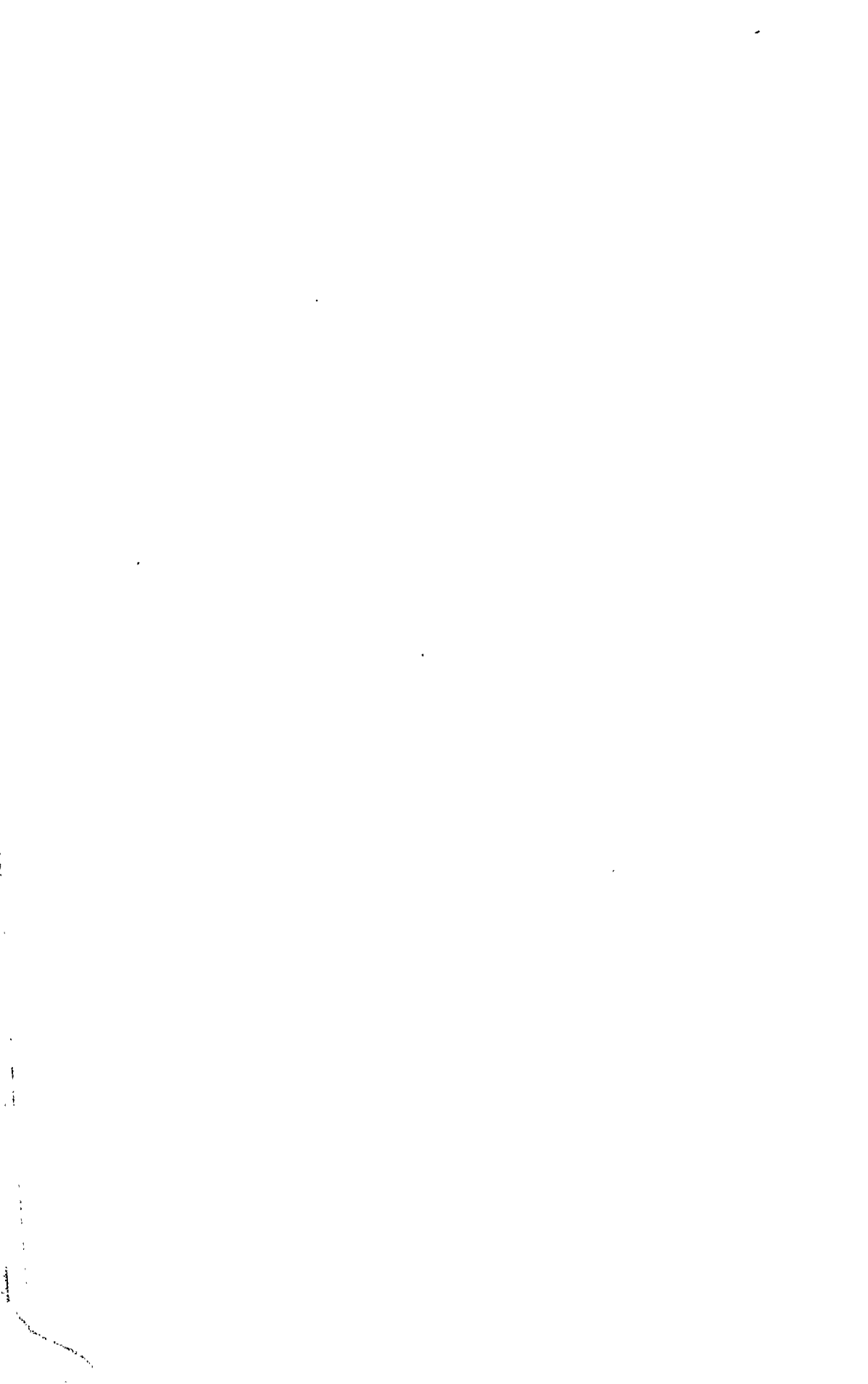
To my wife I express my warmest thanks for her assistance in preparing the drawings.

The investigations have been supported economically by a personal grant from the foundation "Therese och Johan Anderssons Minne", and also by the State Research Committee on Aviation and Naval Medicine.

Stockholm, April 1949.

CARL MAGNUS HESSER.







at all, but rather unilateral. Recent research in this laboratory has clearly demonstrated, that in the earlier stages of hypoxic hyperventilation, one set of chemosensitive cells, *i.e.* the centre, is actually less active than during normal air-breathing. The relationship between centrogenic and chemoreflex drives may convincingly be revealed by observing the respiratory reactions following a sudden withdrawal of all chemoreflex drive, preferably by using the cold-blocking technique. It was found, that the hyperventilatory defense against hypoxia originates entirely in the chemoreceptors, the decrease in central support being dependent on the degree of blood alkalosis or hypocapnia, set up by the purely reflexogenic hyperventilation. The centre may eventually become apneic from missing chemical stimulation during hypoxic hyperventilation. However, apart from the conditions of eupnea and hypoxic hyperventilation, the interaction of centrogenic and reflexogenic chemical control of breathing is very little understood, and has to be studied in other conditions, before the true mechanism of respiratory activity under different physiological and pathological conditions can be explained.

The theme of the present work is to study the rôle of the respiratory centre and the chemoreflex mechanism in the respiratory defense against experimentally induced acid-base displacements in the blood. It seems worth while to inquire into the mutual interplay between the variables in the acid-base balance and the activity of the chemosensitive cells of the centre, 1) when acting alone, and 2) when being modified by the chemoreflex component. Especially when considering that the question of a specific stimulus to the centre itself is still open, and that practically nothing is known about the importance of the chemoreflex component in clinical conditions of acidosis and alkalosis, the need for information on these points should be felt.

An experimental approach to the subject seems particularly justified by 1) the development of the measuring and recording technique, employed in the present work, and 2) the use of the cold-blocking technique. With the aid of special devices for direct, automatic and continuous recording of blood pH and  $p\text{CO}_2$ , two of the three variables in the acid-base balance were

made available for direct observation and control. As a consequence, certain important hemo-respiratory reactions could directly be followed in the anaesthetized dog. It was therefore believed, that some new aspects of an old and complex question — the chemical regulation of breathing — would result from the experimental procedure, employed in the present work.

Before the nature of the main problem is presented (p. 19), a short review is given of certain earlier, basic investigations, from which the present work was derived. However, it was strongly felt, that a vast amount of references had to be omitted for the sake of clarity. No attempt is therefore made to account in detail for the overwhelming mass of data concerning the chemical regulation of breathing, which is now available. Reference will only be made to such investigations, which bear fundamental relationship to the present work. For complete reference the reader should consult special reviews, which will be mentioned in the text.

### III. Approach to the Problem.

#### 1. Physicochemical Factors in the Acid-Base Balance of the Blood.

It is a well known fact that the amount of pulmonary (alveolar) ventilation has a great influence on the acid-base balance of the blood. This fact may now be clearly understood since the classical works of L. J. HENDERSON, HASSELBALCH, HALDANE et al., Y. HENDERSON et al. and VAN SLYKE et al. in the first two decades of this century, from which the modern conception of the acid-base balance in the body is derived. For complete reference, see reviews by LILJESTRAND (1928), PETERS & VAN SLYKE (1931), HALDANE & PRIESTLEY (1935), SHOCK & HASTINGS (1935) and Y. HENDERSON (1938).

It is now well recognized that the acid-base balance of the blood in normal condition as well as in experimental or clinical acidosis and alkalosis can be expressed in terms of three variables, viz. the hydrogen ion concentration,  $[H]$ , the amount of  $CO_2$  in the form of free carbonic acid,  $[H_2CO_3]$ , and the bicarbonate concentration,  $[BHCO_3]$ . In conformity with the HENDERSON-HASSELBALCH equation the relation of these variables

is:  $[H] = K \times \frac{[H_2CO_3]}{[BHCO_3]}$ , in which  $K$  is a constant. The amount

of free carbonic acid is in direct proportion to the  $pCO_2$ , whereas the bicarbonate concentration at a given amount of available alkali varies with the  $pCO_2$  in accordance with the well known  $CO_2$  absorption curves. Since at a given rise in  $pCO_2$  the increase of  $H_2CO_3$  is relatively greater than that of  $BHCO_3$ , it follows that an excess of  $CO_2$  in the blood, *e. g.* during inhalation of  $CO_2$ , will cause an acid shift in the blood reaction ("respiratory acidosis"). On the other hand, a  $CO_2$  deficit, as caused by hyperventilation, will result in alkalosis ("respiratory alkalosis"). When the amount of available alkali is reduced, for instance by accumulation of fixed acids in the

blood, the denominator in the carbonic acid/bicarbonate ratio, and hence the pH, is decreased ("metabolic acidosis"), whereas increased bicarbonate concentration following intravenous injections of alkaline solutions leads to a rise in the pH ("metabolic alkalosis"). The conditions mentioned may secondarily become more or less compensated by changes in respiratory activity and by other adaptive processes, thus restoring pH to normal range.

## 2. Notable Conceptions concerning the Interplay between Acid-Base Displacements in the Blood and the Activity of the Respiratory Mechanism as a Whole.

It is now firmly established that under normal conditions at rest the chemical control of breathing is closely related to the alveolar (arterial)  $p\text{CO}_2$ . This conception is derived from a series of classical experiments by different investigators. MIESCHER-RÜSCH (1885) thus stated, that the  $\text{CO}_2$  percentage in the air of the lungs ordinarily determines the chemical regulation of breathing, and not the  $\text{O}_2$  percentage. The "crossed circulation" experiments on animals by FREDERICQ (1901) showed that alterations in ventilation could be produced by varying the gas composition in the arterial blood supplying the head.

However, it was not until HALDANE & PRIESTLEY (1905) introduced their method for direct determination of alveolar  $p\text{CO}_2$ , that the full significance of carbon dioxide in the control of breathing could be realized. By using this method they were able to demonstrate that, under physiological conditions, the alveolar  $p\text{CO}_2$  is kept almost constant (about 40 mm Hg), and furthermore that an increase of about 0.2 % in the alveolar  $\text{CO}_2$  is sufficient to double the resting alveolar ventilation, whereas a similarly small diminution in the alveolar  $\text{CO}_2$  may produce apnea. When, on the other hand, the oxygen percentage of the inspired gas was changed over short periods, the ventilation was found to remain substantially unchanged until the alveolar oxygen percentage was reduced to about 8. Below this percentage breathing was found to increase markedly with decreasing alveolar  $p\text{O}_2$ . On the basis of these and other ob-

servations the authors stated, that during normal condition the breathing is regulated so as to give a constant percentage of  $\text{CO}_2$  in the alveolar air, and that  $\text{CO}_2$  therefore acts specifically in exciting the respiratory centre. The increased ventilation during pronounced oxygen deficiency was ascribed to a stimulating effect of hypoxemia upon the centre. The exceedingly sensitive response to  $\text{CO}_2$  has later been repeatedly confirmed (see CAMPBELL, DOUGLAS & HOBSON 1914, LILJESTRAND 1918, and NIELSEN 1936).

Later on BOYCOTT & HALDANE (1908) found that the diminution in alveolar  $\text{pCO}_2$  during low atmospheric pressure was caused by hyperventilation due to oxygen deficiency. WALTER (1877) had shown that, when  $\text{HCl}$  was administered to animals by the stomach, the respiration was increased and the bicarbonates of the blood were decreased. Investigations by ARAKI (1894) and GALEOTTI (1904) seemed to show that, during oxygen deficiency, there is a decreased alkalinity of the blood, which was ascribed to excess production of lactic acid. Taking the latter findings into consideration, BOYCOTT & HALDANE, from their observation that the alveolar  $\text{pCO}_2$  does not at once return to normal on returning from low pressure, came to the conclusion, that the combined effect of carbonic acid and other acids on the reaction of the blood is the real stimulus to which the respiratory centre responds.

In 1911 WINTERSTEIN advanced his first "reaction theory", according to which the respiratory activity during all conditions, including oxygen deficiency, is regulated by the arterial  $\text{cH}$ . The theory was strongly supported by the experiments of HASSELBALCH (1912). By changing the diet he could demonstrate considerable alterations in the alveolar  $\text{pCO}_2$ , whereas the arterial  $\text{cH}$  remained substantially unchanged. The blood reaction theory gained practically universal acceptance, and the hyperventilation during oxygen deficiency was explained as caused by "asphyxial acidosis".

However, it was not long before evidence began to accumulate, which seemed to be more or less incompatible with this theory. By using modern electrometric methods for direct measuring of the  $\text{pH}$  of the blood, WINTERSTEIN (1915) could demonstrate that during acute oxygen deficiency the blood

reaction shifts to the alkaline side. LAQUEUR & VERZAR (1912) showed that injections into the aorta of new-born rabbits of acid solutions as well as of weak alkaline solutions with a high  $p\text{CO}_2$  could initiate respiratory activity. They therefore believed that carbonic acid exerts a specific effect independent of its properties as an acid, and that the stimulating effect of the acid solutions was not due to the H-ion, as stated by WINTERSTEIN, but to excessive liberation of  $\text{CO}_2$ ,  $\text{H}_2\text{CO}_3$  or  $\text{HCO}_3^-$  in the tissues. The respiratory response to acetic acid was ascribed also to a specific stimulating effect of the  $\text{COOH}$  ion upon the respiratory centre. HOOKER, WILSON & CONNETT (1917) came to the conclusion, that carbonic acid acts specifically on the respiratory centre. They perfused the isolated medulla of dogs with defibrinated blood of varying  $\text{cH}$  and  $p\text{CO}_2$ , and observed a greater respiratory activity when, at the same  $\text{cH}$ , the  $p\text{CO}_2$  was higher. SCOTT (1918) found no close parallelism between arterial  $\text{pH}$  and respiration, when  $\text{Na}_2\text{CO}_3$  was injected at a slow rate in decerebrated cats. The respiratory response to increased  $\text{CO}_2$  was only somewhat less after the injections. SCOTT stated that undissociated  $\text{CO}_2$  acts as a "specific respiratory hormone" and explained the diminishing in the response to  $\text{CO}_2$  as caused by the fact, that "the threshold value of  $\text{CO}_2$  may be raised in a condition of alkalosis and lowered in the presence of an elevation in the  $\text{cH}$  of the blood". COLLIP and BACKUS (1920) found increased respiratory activity after injection of small amounts of  $\text{NaHCO}_3$  into the cisterna magna or the carotid artery. Intravenous administration of  $\text{NaHCO}_3$  (COLLIP 1920) caused increased ventilation if injected in moderate doses, whereas massive doses caused a gradual decrease in the respiratory movements, which finally ceased. COLLIP deduced a specific sensitivity of the respiratory centre to the  $\text{HCO}_3^-$  ion. MELLANBY (1922) and EGE & HENRIQUES (1926) showed that the respiratory response to increased  $\text{CO}_2$  or oxygen deficiency was much greater than that caused by similar changes in the  $\text{pH}$  of the blood after injection of different acids and alkalis. EGE & HENRIQUES therefore concluded, that  $\text{CO}_2$  exerts a specific action upon the centre.

The most conspicuous contradiction to the old blood acidity theory was raised when HALDANE, KELLAS & KENNAWAY (1919)



and HAGGARD & Y. HENDERSON (1919—20) demonstrated the real nature of the hypocarbic condition during oxygen deficiency. They showed, that the decrease in bicarbonates during hypoxic hyperventilation is not due to an accumulation of acid metabolites, but is secondary to the effects of the hyperventilation. HALDANE, KELLAS & KENNAWAY demonstrated a reduction in excretion of acid and ammonia by the kidneys and concluded, that this should be a quite normal adaptive compensation of the alkalosis produced by the excessive blowing out of  $\text{CO}_2$  during hypoxic hyperventilation. Already in 1918 Y. HENDERSON & HAGGARD (see HENDERSON 1938) had shown in experiments on animals that by merely varying the ventilation of the lungs, and thereby adjusting the  $\text{pCO}_2$  of the blood, they could induce a marked decrease or increase in the alkali as bicarbonates of the blood. When the lungs were over-ventilated, and the  $\text{CO}_2$  in the blood was thus decreased, the bicarbonates were also greatly reduced. These and subsequent observations led them to the conclusion, that the hypocarbic condition during oxygen deficiency is caused by the disappearance of alkali from the blood into the tissues and urine as a result of the hypocapnic condition during hypoxic hyperventilation. Accordingly, the old conception that the decreased "titration alkalinity" of the blood during oxygen deficiency involved acidosis must be given up. From this time on the hypoxic hyperventilation became a real paradox.

In 1921 WINTERSTEIN advanced his second "reaction theory", according to which during oxygen deficiency the chemical regulation of the pulmonary ventilation should be dependent on the  $\text{cH}$  within the respiratory centre itself. Oxygen deficiency was thought to produce a local acidosis in the centre from increased local accumulation of lactic acid. Two years later GESELL (see GESELL 1925) proposed the well-known "intracellular acidity" theory, postulating that "the activity of the respiratory centre is fundamentally a function of its own acidity, as opposed to the acidity of the arterial blood, the specificity of carbon dioxide and the direct stimulating effect of lack of oxygen".

Meanwhile it was shown by JACOBS (1920) that alkaline solutions of  $\text{H}_2\text{CO}_3$  —  $\text{NaHCO}_3$  may exert acid effects in the interior of the cells of certain unicellular organisms and flowers.

The cause of this changing relationship between extracellular and intracellular  $\text{cH}$  was ascribed to a rapid diffusion of  $\text{CO}_2$  into the cell, whereas "the ions as such do not penetrate to any appreciable extent". LOEB (1922) stated, that weak undissociated acids diffuse freely through cell membranes. The increased ventilation following injection of  $\text{NaHCO}_3$  was consequently explained as caused by an acid shift in the centre as opposed to the alkaline shift in the blood. In 1926—27 GESELL and his coworkers (see GESELL 1929) measured the changes in pH in the cerebrospinal liquor and in arterial and venous blood, as well as the changes in the  $\text{CO}_2$  of the expired gas following intravenous injections of different alkaline solutions and pharmaca. Administration of  $\text{NaHCO}_3$  resulted in an alkaline shift in the blood, whereas the liquor turned acid, and the expired  $\text{CO}_2$  increased. These findings were thought to support the intracellular acidity theory.

According to NIELSEN (1936)  $\text{CO}_2$  is "der adäquate Reiz des Atemzentrums", not by virtue of its acid-forming properties, but through a specific action of its own. This conclusion was derived from experiments on human subjects, in which was shown that a certain change in arterial pH was associated with a larger increase in ventilation when caused by inhalation of  $\text{CO}_2$  than by ammonium chloride acidosis. This statement of NIELSEN has been adopted by several authors (cf. KROGH 1941, SCHMIDT 1941, BEST & TAYLOR 1943). The theory of NIELSEN also implies, that alterations in pulmonary ventilation mainly result from changes in the excitability of the centre to  $\text{CO}_2$ . The increased ventilation during ammonium chloride acidosis was consequently explained as caused by an increased excitability of the centre to  $\text{CO}_2$  (see further "Discussion" p. 48).

### **3. Influence of the Discovery of the Chemoreflex Mechanism on the Validity of Earlier Respiratory Theories.**

When J. F. and C. HEYMANS and C. HEYMANS and his collaborators in the years 1924—27 and 1930—32 discovered the peripheral chemoreflex mechanism, a new and more secure foundation was given upon which the physiological research in

respiration could advance. It was not long, until the observations of HEYMANS and his associates became confirmed in the main by a number of different workers, though there still are some difference in opinion about certain points of fundamental importance. During the last years also some new and interesting aspects as to the interaction of centrogenic and chemoreflex drives have been put forth. For reference and reviews see HEYMANS, BOUCKAERT & REGNIERS (1933), EULER & LILJESTRAND (1936), HEYMANS & BOUCKAERT (1939), GESELL (1939, 1941), SCHMIDT & COMROE (1941), BERNTHAL (1944), SCHMIDT (1945), and BJURSTEDT (1946).

The dominating rôle of the chemoreflex drive in the respiratory defense against acute oxygen deficiency is now firmly established. The chemoreflexes are entirely responsible for the hypoxic hyperventilation, at least in the initial stages, that are associated with blood alkalosis. Since the chemosensitive cells of the centre itself have been shown to be less active in hypoxic hyperventilation than during eupnea, the chemoreflexes also have to compensate for the decrease in central support. The latter has been shown to depend directly upon decreased stimulation resulting from hypocapnia and alkalosis, thus being no inhibitory effect from oxygen lack *per se* (BJURSTEDT 1946).

Consequently those theories must be refuted, according to which the hyperventilation during acute oxygen deficiency results from direct stimulating effect of hypoxemia upon the centre (HALDANE & PRIESTLEY 1905), from increased arterial cH (WINTERSTEIN 1911), increased intracental (WINTERSTEIN 1921) or intracellular cH in the centre (GESELL 1923). However, these conceptions may theoretically be as valid to day as they were before the discovery of the chemoreflex mechanism, if restricted to imply that the dominating factor controlling the self-engendered activity of the centre is the  $p\text{CO}_2$  or cH of the arterial blood or of the centre.

GESELL seems to foreshadow the required modification of the original intracellular theory in one of his latest reviews (1941). Stressing the importance of intracellular acid excitation for the activity of the chemoreceptors (see below EULER, LILJESTRAND & ZOTTERMAN 1939) he states, that there is no need for excluding the centre in the general scheme of the intracellular acidity

theory of respiratory control. "Like the chemoreceptors it responds to changes in acidity."

Experiments, showing that the chemoceptively denervated centre responds to increased  $\text{CO}_2$  in the inhaled air, are not actually inconsistent with any of the earlier theories if modified to refer to the chemosensitive cells of the centre. However, only few direct investigations seem to have been performed to elucidate the question as to the identity of the chemical stimulus to the centre itself. BANUS and his collaborators (1944) made intravenous injections of HCl on dogs completely deprived of their chemoreflexes, and found only minimal fluctuations in arterial cH with marked decreases in alveolar  $\text{pCO}_2$ . They therefore attributed the increased pulmonary ventilation to the action of hydrogen ions within the cells of the centre.

The theories, which postulate increased excitability of the centre to the "normal" stimulus during acute oxygen deficiency (LINDHARD 1911, NIELSEN 1936, HENDERSON 1938), seem not to have been experimentally proved. Such an increased excitability is anyhow of minor importance in the acute hypoxic hyperventilation, since during this condition — as mentioned above — the central support in the respiratory defense has later been shown to be markedly reduced.

As to the rôle of the chemoreflex drive in the hypercapnic hyperventilation opinions differ considerably. There is no doubt that the peripheral chemoreceptors are stimulated by increased  $\text{CO}_2$  or cH. This has been shown by studies on the action potentials in the sinus nerves. EULER, LILJESTRAND & ZOTTERMAN (1939) found under condition of pure oxygen inhalation that the chemoceptive fibres began to discharge as soon as the alveolar  $\text{pCO}_2$  exceeded about 30 mm Hg, and that the discharge increased in approximatively linear relation to the  $\text{pCO}_2$ . They also presented evidence that the exciting factor to the chemoreceptors is the cH within the chemoceptive cells themselves.

Increased discharge from the peripheral chemoreceptors during progressive hypercapnia should, however, not be considered the same as increased chemoreflex influence in the respiratory response to hypercapnia. The importance of the chemoreflexes as compared with that of the chemoceptively

isolated centre in the defense against increased  $p\text{CO}_2$  has been much discussed with respect to 1) sensitivity (threshold for  $\text{CO}_2$  stimulation), 2) relative power of the two components, and 3) speed of reaction. The experimental results have unfortunately been controversial in all three respects, and as yet the questions must be considered open. Conceivably the differences in experimental approach have largely contributed to the confusion. In several cases the carotid bodies have been separately perfused, and from these experiments it is quite clear, that hypercapnic and weak acid solutions may produce reflex hyperventilation, when acting locally on the chemoreceptors. However, quantitative data as to the nature of the reflex stimulation can hardly be obtained from studies of the amount of ventilation in perfusion experiments, since the respiratory reactions also depend on the condition of the centre. For instance, isolated stimulation of the peripheral chemoreceptors with resulting hyperventilation will produce hypocapnia and alkalosis at the centre, which in turn will diminish the central component, thus modifying the outcome of chemoreceptor excitation (cf. HEYMANS & BOUCKAERT 1939). Hence, as long as the stimulation of chemosensitive cells in the chemoreceptors and centre is not caused by chemical changes in the arterial blood, acting on both structures, the condition is not only artificial but will derange the normal interaction of the two components.

In this connection some interesting experiments by GESELL, LAPIDES & LEVIN (1940) seem to have given new aspects on the chemoreflex mechanism. By repeated cold-blockings of the sinus nerves during progressive hypercapnia they found that, as hypercapnia was becoming more intense, temporary withdrawal of the chemoreflex component caused a diminishing absolute reduction of the total ventilation. At 5—6 per cent of  $\text{CO}_2$  in the inspired air blocking no more caused any effect whatever. It was concluded that increasing  $p\text{CO}_2$  at the centre or elsewhere along the chemoreflex pathway exerts an increasing blocking effect on the signals set up in the chemoreceptors, and that hyperpnea of high grade hypercapnia is purely centrogenic.

#### 4. The Problem.

From the investigations mentioned in the preceding review it is evident, that the question is still open as to the "identity of the chemical stimulus" for the self-engendered activity of the respiratory centre itself. Among the variables in the acid-base balance, which is without doubt of very great importance in the respiratory adjustments to the metabolic needs of the body, both  $\text{cH}$  and the  $\text{CO}_2$  molecule are known to exert a stimulating influence upon the activity of the chemoceptively denervated centre. However, there are differences in opinion, as to 1) which of these variables in the acid-base balance is the dominant factor, and 2) the mode of action of these variables. Very little is known concerning the interaction between central and chemoreflex components in the respiratory activity during acid-base displacements under high arterial oxygen saturation. Both questions are of major importance for the understanding of the chemical control of the respiratory apparatus as a whole. It seems therefore pertinent to inquire into the above-mentioned mechanisms in the trend to elucidate the various factors, which are involved in the complex regulation of the pulmonary ventilation.

The response of the respiratory centre itself to changes in its chemical milieu can be studied in animal experiments by observing respiratory reactions after complete, surgical disconnection of the chemoreflex mechanism. It therefore seemed probable that, by using a procedure, in which the chemoceptively denervated centre could be studied under the influence of experimentally induced acid-base displacements in the blood, as judged by the aid of a special measuring and recording system, some new information on the chemical excitatory mechanism of the centre itself could be gained.

Information could also be expected on the importance of the chemoreflexes in the respiratory defense against acid-base displacements by use of the same experimental facilities in the chemoceptively intact animal in combination with the cold-blocking technique. The chemoreflex component cannot be studied by disconnection of the centre, since the latter is a necessary integrant in the nervous pathway from the chemo-

receptors to the respiratory muscles. However, by suddenly and temporarily blocking the chemoreflex drive, the relationship between central and chemoreflex components in the respiratory activity is promptly revealed, at a given situation of interaction.

It should be stressed at once, that the cold-blocking experiments must not be contaminated with such experiments, in which respiratory reactions are observed after permanent chemoreflex denervation, made in advance. This is because the result of a "chronical" denervation will conceivably be a condition, which entirely differs from that which is set up by the normally continuous chemoreflex influence upon the centre. Neither can such respiratory reactions be considered representative for the absolute response of the peripheral chemosensitive structures, which are obtained in experiments with isolated perfusion of these regions, since the reflex influence on the respiratory muscles are greatly modified by chemical changes, induced at the centre by the respiratory reactions *per se*.

The above-mentioned experimental approach to the problem of the importance of the chemoreflexes in acidotic and alkalotic conditions, *i.e.* the cold-blocking technique, was therefore considered the method of choice.

## IV. Experimental Section.

### 1. Methods.

#### General Procedure.

All experiments were performed on dogs under chloralose anaesthesia (see below "Anaesthesia"). 32 dogs of 12 to 32 kilos weight were used.

One principal feature of the method involved direct, simultaneous and continuous measuring and recording of the relative changes in pulmonary ventilation, alveolar carbon dioxide pressure and arterial pH (see below "Measuring and Recording Apparatus") during the development of experimentally induced acidotic and alkalotic conditions. Another integrant was to reveal experimentally the centrogenic and chemoreflex components at different stages of such conditions.

Deviations from the normal acid-base balance were induced either by having the animals inspire a mixture of  $\text{CO}_2$  in  $\text{O}_2$ , or by intravenous injections of  $\text{HCl}$  and  $\text{NaHCO}_3$ , as well as  $\text{Na}_2\text{CO}_3$ . The reason for choosing  $\text{HCl}$  in favour of those organic acids, which are actually formed by the body itself under certain conditions (*e. g.* lactic and acetic acid), was that the latter acids are normally oxidized in the body, thereby inducing only a transient acidotic displacement. Since preliminary observations revealed that the hemo-respiratory reactions following the injections were highly dependent on the rate of injection, these were usually performed by means of a special syringe assembly (see below "Administration of Acid and Alkaline Solutions"), admitting an administration of the solutions at a desired and constant rate.

In a first series of experiments the hemo-respiratory reactions under acid-base displacements were studied in animals, the respiratory centre of which had been permanently freed from all known influences from peripheral pressoreceptor and chemoreceptor excitation (see below "Disclosing of Centrogenic and Chemo-



reflex Components"). To exclude any devitalizing effect of hypoxemia upon the centre, the animals were allowed to inhale continuously oxygen-rich gas mixtures.

In order to reveal the centrogenic and chemoreflex components in the respiratory activity under acid-base displacements in the blood, the chemosensory impulses in the sinus nerves were temporarily cold-blocked at different stages of such conditions (vagi and pressosensory fibres originating in the sinuses being cut. See below "Disclosing of Centrogenic and Chemoreflex Components"). Hence, when blocking the impulses in the sinus nerves all known remaining chemoreflex influences were selectively eliminated. Thus any change in respiratory activity on blocking and deblocking must be referred to withdrawal and addition of the remaining chemoreflex drives.

However, the possibility of an actual excitation of the sinus nerves during cooling had to be taken into account. As shown recently by EULER (1947) a local cooling down to about 15° C. of the mammalian somatic nerves by means of the thermode technique excites the thick myelinated afferent fibres but not the thinnest afferents. Though in the present work the sinus nerves were rapidly cooled locally to about 0° C., there may conceivably be some adjacent parts of the nerves central to the thermodes which were only slowly cooled down to about 15° C. as in the experiments made by EULER. The electroneurogram of the sinus nerves of the dog has been found to consist of larger, medium-sized and very small action potentials, the first two groups deriving from baroreceptive fibres only, the latter also from chemoceptive fibres (EULER & ZOTTERMAN 1942). Therefore no theoretical evidence of excitation of the chemoceptive fibres during cooling of the sinus nerves seems to exist (nor have any signs of such excitation been revealed in the present work). As to the pressosensory fibres the assumption of such a cooling excitatory effect may be warranted. Actually an initial decrease in the arterial blood pressure may be observed on blocking the sinus nerves, after the vagi and baroreceptive fibres originating in the sinuses have been sectioned. However, this "depressor" effect was most striking when blocking the sinus nerves during hypoxemia. This effect may therefore readily be explained as caused by a withdrawal of a pressor drive from the chemore-

ceptors (cf. EULER & LILJESTRAND 1942). In addition, blocking with the pressosensory fibres intact always caused a large increase in the arterial blood pressure, indicating that the pressosensory fibres were not excited to any significant degree, but that in these cases the pressor impulses were actually blocked.

It was therefore concluded that the cold-blockings as used in the present work actually caused a functional, temporary, but complete disconnection of the remaining chemoreflex component in the respiratory activity.

It was considered important to avoid changes in the respiratory activity from fluctuations in the body temperature. Therefore the body was maintained at practically constant temperature by means of built-in heaters in the operating table. The body temperature was checked with a thermometer in contact with the circulating blood (see below "Arterial pH").

### Anaesthesia.

In all experiments chloralose was used, administered intravenously in doses of about 10 ml per kg of a 1 per cent solution. In order to avoid spontaneous changes in the respiratory activity during the course of an experiment as a result of alterations in depth of anaesthesia, small succeeding doses of chloralose were given at intervals of 2—3 hours. The anaesthesia level was controlled so as to just suppress spinal motor reflex reactions.

### Operation.

A special cannula belonging to a device for automatic recording of alveolar  $p\text{CO}_2$  (see below "Alveolar Carbon Dioxide Pressure") was inserted into the trachea. The right femoral vein was used for intravenous injections, while the right femoral artery was used for recording of the arterial blood pressure. On those dogs, on which the cold-blocking experiments were performed, the carotid regions were exposed and the pressosensory fibres from the sinus regions severed, essentially in accordance with the general technique previously described and discussed in detail by BJURSTEDT (1946). The following modifications of this technique were employed. Before the pressosensory denervation began large doses of heparin (about 5 mg/kg) were given intravenously in order to avoid the risk of clotting in the carotid bodies, which might have otherwise occurred when operation in these regions was carried out. The most painstaking ligation of all open vessels in the operation wounds therefore had to be done in order to avoid large losses

of blood through hemorrhage. The pressosensory fibres from the sinus regions were then severed before by-passing the carotid bodies by means of a fine pair of scissors. However, in several instances it was found advantageous not to cut the pressor fibres altogether but to ligate a large portion of them in order to avoid damage of small arteries which frequently run amidst the pressor fibres. By denuding the walls of the sinuses and the large vessels near the bifurcation the nerve network on the surfaces of these vessels was destroyed. After the vagi had been cut between ligatures in the mid cervical region the pressosensory denervation was considered complete when no pressor effect appeared after clamping of the common carotids. To avoid injury of the sinus nerves a thin layer of tissue surrounding the nerves was left. Finally the glass chamber belonging to the devices for measuring and recording of the arterial pH and oxygen saturation (see below "Arterial pH" and "Arterial Oxygen Saturation") was inserted into the left femoral artery.

### **Disclosing of Centrogenic and Chemoreflex Components.**

Temporary blockings (usually lasting 1–2 minutes) of the chemosensory impulses in the sinus nerves were attained by local and temporary cooling of the sinus nerves. For this purpose fine silver thermodes were placed around the tissue cords containing the sinus nerves (see "Operation"), about a half to one centimeter central to the carotid bodies. The temperature of the thermodes could be decreased to about  $0^{\circ}\text{C}$ . within a few seconds by means of rapidly circulating alcohol of  $0^{\circ}$  to  $-2^{\circ}\text{C}$ ., sucked from a large container in a cold-store refrigerator. Deblocking could be brought about within a few seconds by shifting to alcohol of  $37\text{--}38^{\circ}\text{C}$ ., obtained from a large container including a thermo-relay arrangement. Usually the thermodes were permanently kept in place during the course of an experiment, the head of the dog being fixed to the operating table by means of a clamp. After the vagi had been cut the effectiveness of the cold-blocking was repeatedly tested by checking that no respiratory stimulation was evoked by inhalation of 8–10 per cent  $\text{O}_2$  in  $\text{N}_2$  or by intravenous injections of chemoreflexly active doses of NaCN. The general vitality of the chemoreflexes on deblocking was checked in the same manner.

When a permanent elimination of the presso- and chemoreceptor reflexes was desired, the vagi were cut between ligatures, and the tissue-cords containing the sinus nerves ligated about one centimeter central to the carotid bodies. The effectiveness of the denervation operation was tested in the same manner as when testing the effectiveness of the cold-blocking (cf. fig. 4 D).

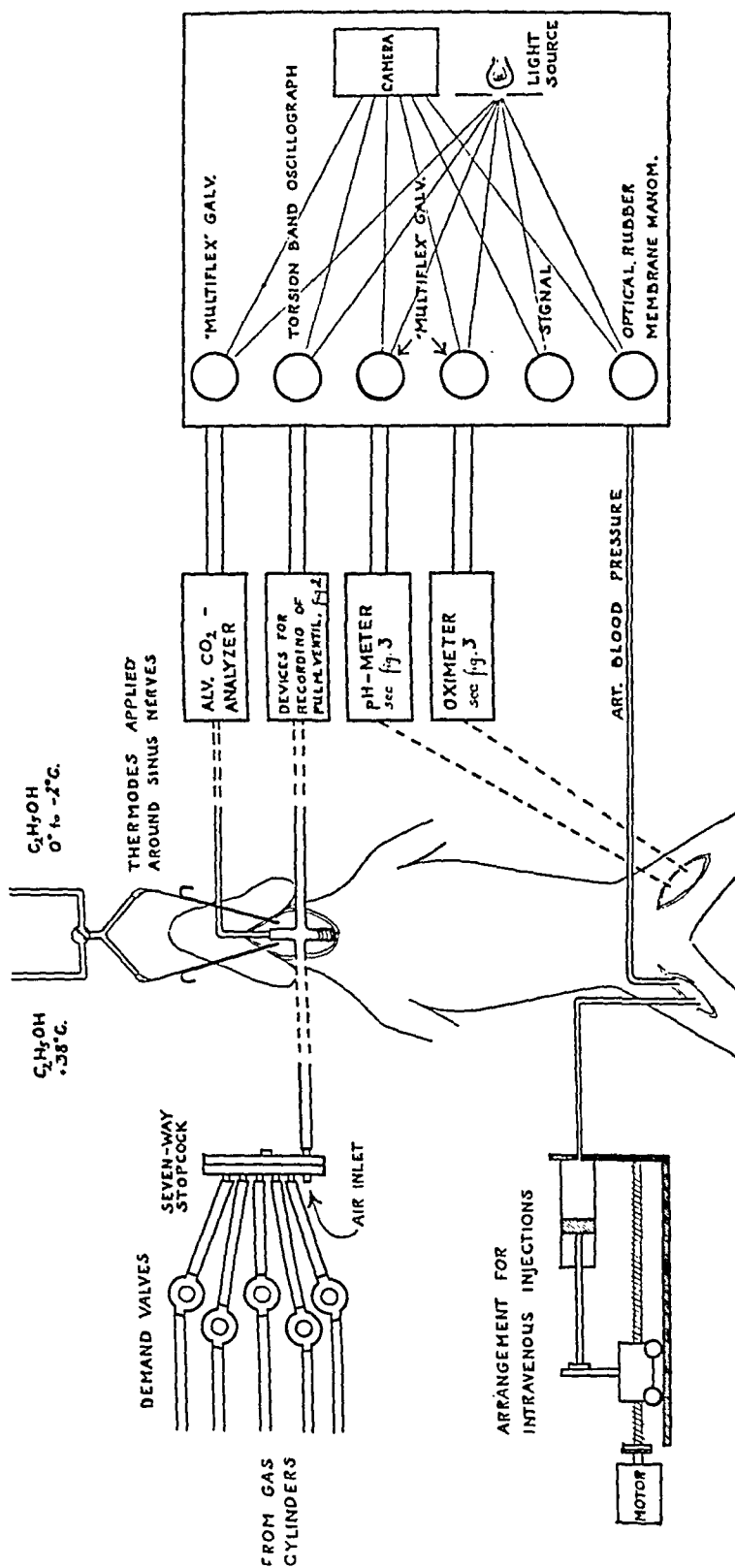


Fig. 1. General scheme of experimental arrangement.

## Measuring and Recording Apparatus.

For the present work a number of devices have been constructed for measuring and recording directly, automatically and continuously a) the pulmonary ventilation, b) the alveolar carbon dioxide pressure, c) the arterial pH, d) the arterial oxygen saturation, and e) the systemic arterial blood pressure. These variables were recorded simultaneously on photographic paper by means of an optical system and a camera. Fig. 1 gives a schematic representation of the general arrangements of the measuring and recording systems.

### a) *Pulmonary Ventilation.*

The volume of breathing was recorded by using alternatively two different methods (fig. 2). In one series of experiments the animals were allowed to rebreathe in a Krogh's respirometer in which the soda lime was removed in case a gradual increase of the  $\text{CO}_2$  concentration of the inspired gas was desired. In order to prevent a gradual and technically disturbing sinking of the respirometer lid caused by oxygen consumption or removal of gas through the  $\text{CO}_2$ -analyzer (see below "Alveolar Carbon Dioxide Pressure"), the closed system was automatically supplied with additional oxygen as corresponding to the loss of gas. This was made by having the oxygen, which was stored under pressure in a steel cylinder, pass a pressure reducing valve and then an electromagnetic valve before entering the respirometer. Each time the lid would sink to a certain level a contact arrangement was closed and power supplied to the electromagnet. This opened the valve temporarily and the respirometer was thus inflated with small amounts of oxygen. The oxygen pressure of the inspired gas could be checked by means of a Pauling oxygen tensimeter. The movements of the respirometer lid were transmitted to a sliding aluminium screen, the latter uncovering more or less the light-sensitive surface of the phototube of an electronic light-measuring device. After passing a direct current 1-stage vacuum tube amplifier the changes of the phototube current, caused by the movements of the respirometer lid, were directly recorded on photographic paper by means of a torsion band oscillograph.

In another series of experiments, in which the composition of the inspired gas had to be changed suddenly and then kept constant for a certain period, the expiratory volumes were recorded in the following manner. The gas mixtures needed for inhalation were delivered from large steel cylinders, containing 40 liters of gas at a pressure of about 150 atmospheres. Each gas mixture first passed a pressure reducing valve and then a demand valve, Pioneer type A-12 regulator. The outlets of the demand valves

were connected to the six openings on the inlet-side of a seven-way stopcock. The stopcock consisted of a slide valve formed by two pieces of brass with a centre bolt. One of the plates was provided with six holes and the other with a single opening. The latter was connected to the inspiratory side of a one-way respiratory valve. Shifting from one gas mixture to another could be made quickly by turning the slide valve during the expiratory phase to the desired position. Since the volume of the gas in the tube connection between the slide valve and the trachea was relatively small, the actual shifting of inspired gas usually took place during the subsequent inspiratory stroke. The expiratory side of the respiratory valve was connected to a gas meter. By means of a friction coupling, the turning of the meter spindle with each expiration was transmitted to an iron shaft placed along the axis of a coil electromagnet. A cord wound on a brass wheel on the iron shaft was fastened to the screen of the afore-mentioned phototube device. In this way the screen moved in proportion to the volume of each expiration. At certain intervals power was automatically supplied for a short period to the electromagnet from a time-controlled relay. This caused a temporary release of the friction coupling which allowed the spring-loaded shaft and screen to return rapidly to their zero setting. In this way the volume of breathing was recorded on the photographic paper as a stair curve, the height of each step corresponding to the volume of each expiratory stroke and the height of an entire stair to the volume of expired gas during a certain period of time.

#### b) *Alveolar Carbon Dioxide Pressure.*

The alveolar  $p\text{CO}_2$  was measured and recorded automatically and continuously by means of a method especially developed for the present purpose and described in detail in the "Appendix". Alveolar samples were drawn automatically from the trachea of the animals only during the last phase of each expiration and the subsequent respiratory pause, irrespectively of rate and depth of breathing as well as of resting respiratory level. The carbon dioxide in the intermittently obtained samples was measured and recorded automatically and continuously by a thermal conductive analyzer, especially designed for shortness of time lag and automatic compensation of disturbances from co-existent gases.

#### c) *Arterial pH.*

The pH of the circulating arterial blood was measured electrometrically by means of a glass electrode assembly (see fig. 3) inserted into the left femoral artery, and a special tube electro-

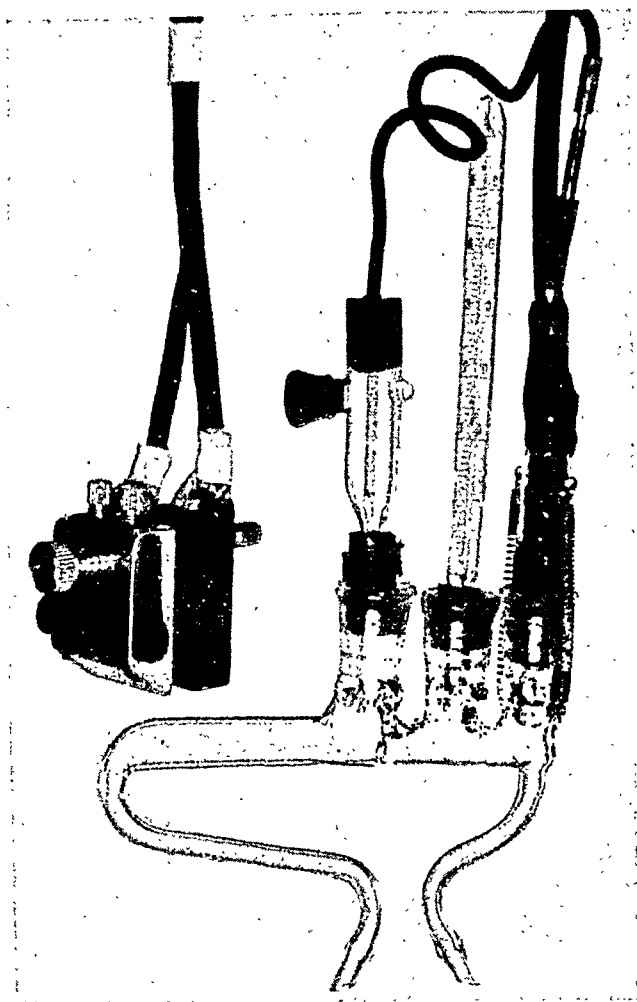


Fig. 3. Oximeter and glass electrode assembly (half natural size).

meter, previously described by BJURSTEDT (1946). The device was especially designed for suppression of zero drift, the latter amounting only to some millivolt during uninterrupted measuring of up to 10 hours. A Multiflex galvanometer, type MG 3, connected to the electrometer continuously recorded the changes in arterial pH with a time lag of about one second. By switching temporarily the glass and reference electrodes to a BECKMAN pH-meter, model M, momentary pH-values could be read on the dial of the latter. The galvanometer deflexion corresponding to 0.1 of a pH-unit could be checked and photographed by switching in a special calibrator. The temperature of the circulating blood was checked by means of a small thermometer, fitted into the glass electrode chamber. Clotting was prevented by repeated intravenous injections of large doses of heparin.

#### d) *Arterial Oxygen Saturation.*

The oxygen saturation of the circulating arterial blood was measured and recorded continuously by means of a photo-electric oximeter device, automatically compensating for alterations in the hemoglobin content of the circulating blood. The principles and theory of the compensating oximeter have been described by MILLIKAN (1942). The oximeter unit was placed over a flattened prolongation of the glass electrode chamber (see fig. 3), the interior transverse section of the prolongation amounting to about  $1.0 \times 8$  mm. In this way a constant and homogeneous optical medium between the lamp and the barrier-layer light-sensitive cells of the oximeter was ensured, disregarding the variations in the hemoglobin content of the circulating blood. However, by using an especially elaborated modification of the "third" MILLIKAN oximeter device the changes in light-transmission owing to dilution of the circulating blood were automatically compensated. The oximeter device was connected to a Multiflex galvanometer, type MG 3, the sensitivity of which could be varied by means of a rheostat. The calibration of the oximeter was made principally according to MILLIKAN. The "COLEMAN filter A" was used for adjustment of the "green" (infra-red) light-transmission, whereas 100 % oxygen saturation was attained by having the dog breathe pure oxygen or carbogen (6.5 %  $\text{CO}_2$  in  $\text{O}_2$ ). The glass electrode and the thermometer of the glass chamber were placed proximal to the oximeter unit in order not to be influenced by the heat from the lamp of the oximeter.

#### e) *Systemic Arterial Blood Pressure.*

The arterial blood pressure was recorded from the right femoral artery by means of an ordinary optical rubber membrane manometer. The tube connecting the artery cannula and the manometer was filled with Ringer solution to which had been added heparin in order to prevent clotting of blood streaming out into this tube connection.

### **Administration of Acid and Alkaline Solutions.**

Conditions simulating metabolic acidosis and alkalosis were experimentally induced by giving intravenously solutions of  $\text{N}/2$   $\text{HCl}$  and  $\text{NaHCO}_3$  as well as  $\text{Na}_2\text{CO}_3$ , the bases usually in molar concentrations. The  $\text{NaHCO}_3$  solutions were always freshly prepared and used immediately in order to avoid transition to  $\text{Na}_2\text{CO}_3$  by escape of  $\text{CO}_2$ . The solutions were administered in varying doses and at different rates. When a constant rate of injection was desired the plunger of the syringe was uniformly pushed by means of a motor-driven propelling screw (see fig. 1).



## 2. Preliminary Observations and Comments.

Before the main results are described and discussed (p. 39) some preliminary observations which are only indirectly related to the main theme of the present work will be mentioned and commented on. Some of these observations deal with questions connected with the procedures and technique used for ensuring uniformity of the experimental basic conditions. Other preliminary observations refer more closely to the main problem and had to be considered in the interpretation of the main results.

### **Influence of Anaesthesia and Operation.**

It has been stated by SCHMIDT (1940) that the use of chloralose as an anaesthetic disturbs the normal interrelationship between centrogenic and chemoreflex components in the respiratory activity not only by causing a depression of the central response to carbon dioxide but also by actually exaggerating the chemoreflex excitability. It seems conceivable that, in order to moderate this unbalance between centrogenic and chemoreflex drives, the anaesthesia should be light. However, in the too lightly anaesthetized animal an increased respiratory activity may incidentally be evoked reflexly from sensory, non-specific stimulation. If chloralose is used in suitable doses major disturbances may be avoided, whereas at the same time the depression of the respiratory centre may be fairly insignificant as indicated by the absence of a rise in the alveolar carbon dioxide pressure (see EULER & LILJESTRAND 1936, 1940). EULER & LILJESTRAND further showed that under such conditions a fairly stable state in respect to respiration could be kept for hours as revealed by the relative constancy of the alveolar carbon dioxide pressure.

The disturbed balance between centrogenic and chemoreflex components in eupneic breathing under chloralose anaesthesia seems to be an established fact. This unbalance may partly be explained by an increased hypoxic excitation of the peripheral chemoreceptors owing to a certain degree of desaturation of the arterial blood, which usually develops under such conditions. A simultaneous depression of the centrogenic drive may therefore be masked. A certain depression of the central response to  $\text{CO}_2$  could actually be demonstrated in the present

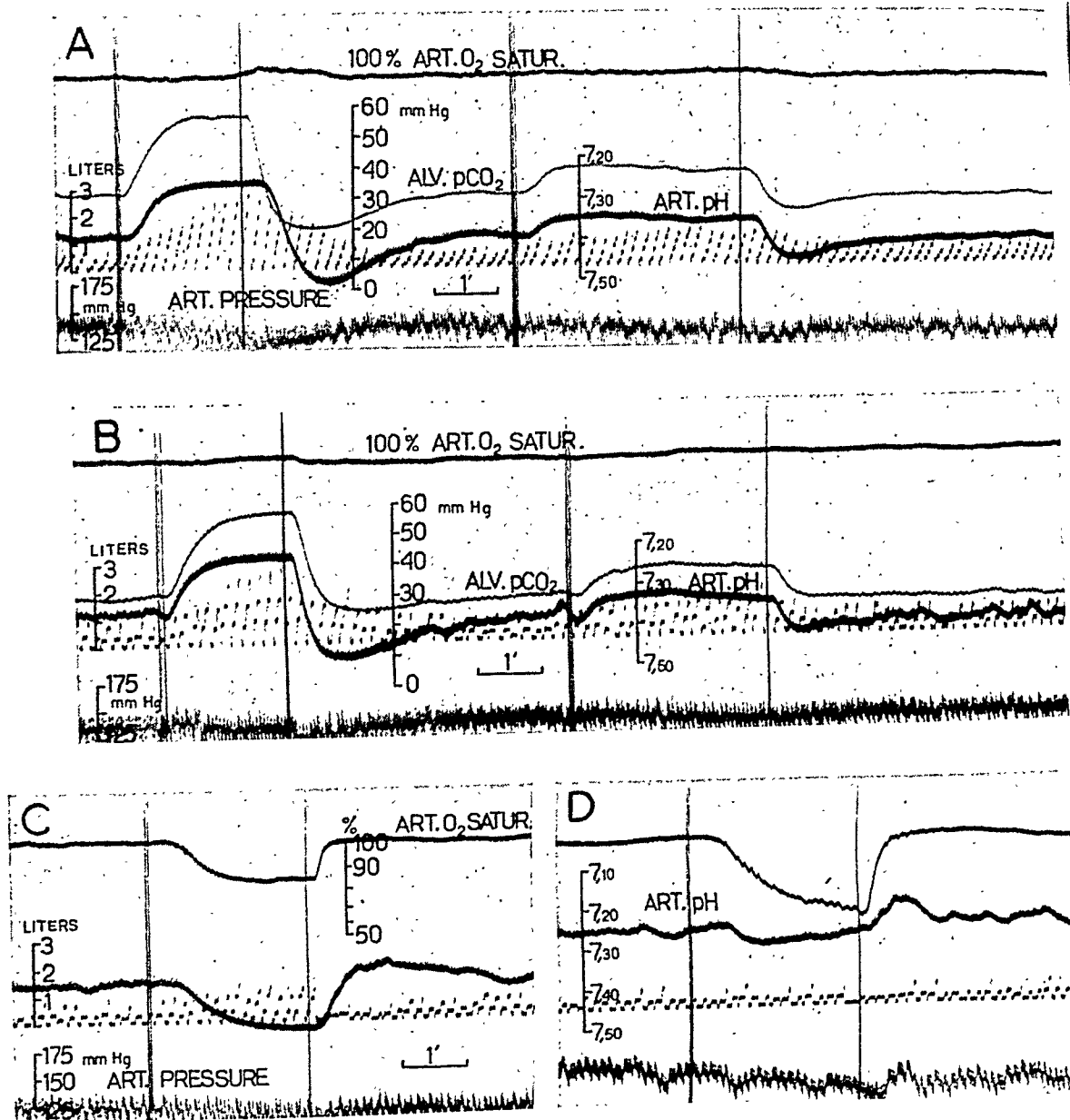


Fig. 4. A and B. Effects of sudden shifts from pure oxygen to CO<sub>2</sub>-O<sub>2</sub>-mixtures in chemoceptively intact dog. Between A and B section of the vagi. Note the lacking parallelism between pulmonary ventilation and arterial pH. No respiratory insufficiency after bilateral vagotomy during high arterial O<sub>2</sub> saturation, although the respiratory pattern has changed. Between first two markings 6.5 % CO<sub>2</sub> in O<sub>2</sub>, between following two markings 2.8 % CO<sub>2</sub> in O<sub>2</sub>.

C and D. Reactions on administration of 7.9 % O<sub>2</sub> in N<sub>2</sub> instead of pure oxygen before (C) and after (D) denervation of the sinuses (vagi cut), illustrating the mutual relationship between O<sub>2</sub> saturation and pH of the blood. Note: in D after the shift to the oxygen-poor gas mixture the pH-tracing goes towards the alkaline side although no increase in ventilation occurs. When shifting back to pure oxygen, however, the pH-tracing shows a shift to the acid side again without any simultaneous decrease of ventilation.

experiments by observing the hemo-respiratory reactions of the anaesthetized dog when shifting from  $O_2$  to mixtures of  $CO_2$  in  $O_2$  in the inspired gas (see fig. 4 A). Though the increase in pulmonary ventilation was marked, the same rise in alveolar  $pCO_2$  or arterial  $cH$  would presumably have evoked a larger increase in the unanaesthetized animal.

It would be suspected, that if the chemoreflex excitability is actually exaggerated in chloralose anaesthesia, the chemoreflex drive elicited by  $CO_2$  or arterial  $cH$  should increase relative to the centrogenic drive, the deeper the anaesthesia. In the experiment illustrated in fig. 5, the respiratory apparatus was profoundly depressed by adding large doses of chloralose. In consequence the ventilation decreased considerably and the  $pCO_2$  and  $cH$  of the arterial blood increased correspondingly, whereas shifting from  $O_2$  to 2.8 %  $CO_2$  in  $O_2$  in the inspired gas hardly evoked any increase in the respiratory activity. The relative importance of the centrogenic and chemoreflex drive in this condition was revealed when the chemoreflexes were blocked. A just observable increase in the average arterial  $cH$  (indicating a very small decrease in the alveolar ventilation) during the block points to the minor importance of the chemoreflex component and to the dominant rôle of the centrogenic support. It is therefore concluded that chloralose only to an insignificant degree, if at all, exaggerates the chemoreflex drive, caused by  $CO_2$  or arterial  $cH$ .

Since a spontaneous change in the depth of anaesthesia may result in a changed balance between central and chemoreflex components, it was considered important to keep the anaesthesia level as constant as possible. As already mentioned a fairly stable state in respect to respiration may be kept for hours if chloralose is given in suitable doses. By additional small doses of chloralose at intervals of some hours this condition could usually be extended to last during the entire course of an experiment. In the present experiments it was therefore no obvious reason to expect any significant change in the balance between centrogenic and chemoreflex drives due to a mere change in the depth of anaesthesia. It is therefore concluded that changes in this balance, as revealed in short-lasting cold-blocking experiments under different acid-base displacements

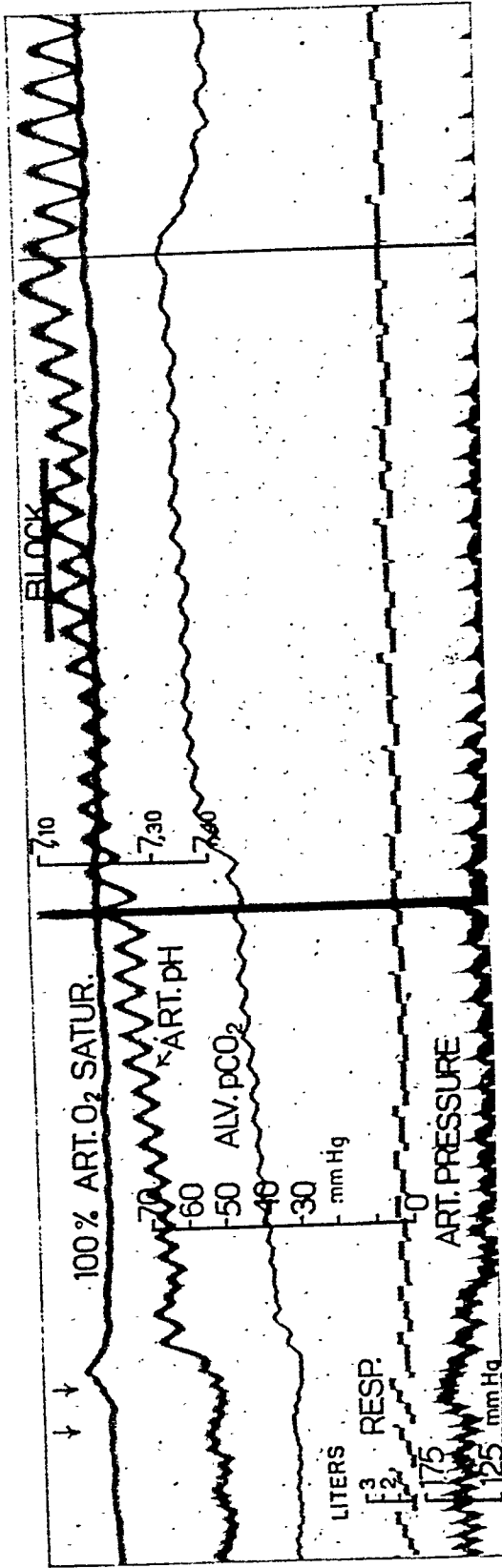


Fig. 5. Changes in the hemo-respiratory functions following increased depth of chloralose anaesthesia. Between  $\downarrow\downarrow$  intravenous injection of 30 mg chloralose per kilo. Ventilation decreases and consequently arterial cH and alveolar pCO<sub>2</sub> increase. Administration of 2.8 % CO<sub>2</sub> in O<sub>2</sub> instead of pure oxygen between vertical markings reveals only a minor respiratory response. Blocking of the chemosensory impulses produces only insignificant change in ventilation as revealed by the arterial cH-tracing. This illustrates that the impulses deriving from the chemoreceptors during CO<sub>2</sub>-breathing have insignificant importance for the respiratory activity even during deep chloralose anaesthesia. Note the marked fluctuations in the arterial cH when the rate of breathing becomes slow.

in the blood, refer to actual changes in the mutual relationship between central and chemoreflex components.

It was often found that after the preparatory operative procedure had been completed under air breathing, the alveolar  $p\text{CO}_2$  was rather low. At the same time the arterial pH was found to be either "normal" or swung to the acid side, implying a decrease in the  $\text{BHCO}_3$  content of the blood. The cause of the hypocarbic condition was here presumably complex. The condition may have involved a process, in which alkali was "driven out of the blood" in consequence of the prevailing hypocapnia (see HENDERSON 1938). The hypocapnia, on the other hand, may have been the result of decreased metabolic production of  $\text{CO}_2$  under narcosis (HENDERSON 1938) and increased ventilation. Several factors may have cooperated to produce increased ventilation, viz. 1) initial excitation from the anaesthetic per se, 2) stimulation from surgical trauma, and 3) exaggerated hypoxic stimulation of the chemoreflexes under chloralose anaesthesia and air breathing. Apart from the secondary condition of alkali being driven out of the blood, a decrease in the  $\text{BHCO}_3$  content of the blood may possibly also result from liberation of acid metabolites from injured regions.

After the operation had been almost completed, oxygen-rich gas mixtures were usually given throughout the experiment. After shifting to increased oxygen the vagi were sectioned in the mid cervical region in order to eliminate any influence from the aortic pressore- and chemoreceptors. Here it was necessary to consider the statement that vagotomy leads to respiratory insufficiency (cf. HESS 1931, GOLLWITZER-MEIER & LERCHE 1940). However, as judged from the alveolar  $p\text{CO}_2$ , the alveolar ventilation remained practically unchanged after double vagotomy during high oxygen (fig. 4 A and B), although the respiratory pattern changed to a deeper and less frequent type, often with a considerable change in the pulmonary minute volume.

### **Arterial (Alveolar) Carbon Dioxide Pressure and pH.**

It is well known that as long as the metabolic production of  $\text{CO}_2$  remains unchanged and a "steady state" prevails, determination of the average alveolar  $p\text{CO}_2$  yields a more satis-

factory and direct information as to the effectiveness of the respiratory activity than does measuring of the pulmonary ventilation. An increase or decrease in the alveolar  $p\text{CO}_2$  under these conditions proves that the alveolar ventilation has decreased or increased.

Since the  $p\text{CO}_2$  of the alveolar air is usually practically the same as in the systemic arterial blood (KROGH & KROGH 1910, Bock et al., 1929), it follows from the HENDERSON-HASSELBALCH equation that, under an unchanged buffering capacity of the blood, the  $\text{cH}$  of the arterial blood will change parallel to the alveolar  $p\text{CO}_2$ . Hence, a continuous recording of arterial  $\text{pH}$  and/or alveolar  $p\text{CO}_2$  will reveal even the slightest changes in the alveolar ventilation and thus in the effectiveness of the respiratory activity, also when no significant changes in pulmonary ventilation can be detected directly. In some of the present experiments, especially in those with cold-blocking of the sinus nerves, the respiratory activity was therefore sometimes preferably judged from the alveolar  $p\text{CO}_2$  and/or arterial  $\text{pH}$ .

It should be quite difficult to determine directly and continuously the variations in the alveolar  $p\text{CO}_2$  within the respiratory cycle itself (see "*Appendix*"). However, this can be made indirectly by recording continuously the  $\text{pH}$  of the arterial blood, as has been done in the present work. The rather paradoxical fact was established that the fluctuations in the arterial  $\text{pH}$  during the course of a respiratory cycle increased with the buffering capacity of the blood as alkali was given intravenously. The observation is, however, not so surprising when remembering that the  $\text{BHCO}_3$  fraction has little buffering influence against carbonic acid as compared to stronger acids. The main point is further, that when alkali is given, the alveolar  $p\text{CO}_2$  also increases. Hence, the pressure difference between alveolar  $p\text{CO}_2$  and the  $p\text{CO}_2$  of the ambient air is increased. Therefore great fluctuations in the alveolar  $p\text{CO}_2$  appear with each inspiration, further exaggerated by the slow rate of respiration.

Also in other cases in which for some reason the rate of respiration was appreciably decreased (*e.g.* in vagotomized animals), considerable fluctuations could be observed in the

saturation and pH of the arterial blood is illustrated in fig. 4 D. A sudden shift from  $O_2$  to inspiration of 7.9 %  $O_2$  in  $N_2$  in the completely presso- and chemoceptively denervated dog caused a marked hypoxemia, whilst the pulmonary ventilation gradually decreased. Meanwhile the arterial pH shifted to the alkaline side in consequence of the oxygen desaturation, but then slowly decreased as a result of the decreasing ventilation. When the dog was suddenly allowed to breathe pure oxygen again, the arterial blood became rapidly saturated with oxygen. As a result the blood became more acid until the ventilation began to increase again, which caused the arterial pH to approach gradually its pre-hypoxic level.

### **Systemic Arterial Blood Pressure and Pulmonary Ventilation.**

The influence of changes in the arterial blood pressure upon the respiratory activity had to be recognized especially in those experiments in which rapid injections of acid or alkali were made. In the completely "presso-denervated" animals changes in the arterial pressure may alter the respiratory activity in consequence of alterations in blood perfusion of the centre (cf. SCHMIDT 1941) or of the carotid bodies (EULER & LILJESTRAND 1936, 1937, 1940, EULER, LILJESTRAND & ZOTTERMAN 1939, BJURSTEDT & HESSER 1942). The presence of intact pressor reflexes in certain injection experiments, on the other hand, puts forth the question as to the possibility of a reflex inhibition of the respiratory activity from an increase in the arterial blood pressure (cf. HEYMANS & BOUCKAERT 1930, KOCH & MARK 1931, BJURSTEDT & EULER 1942). However, for the interpretation of the results of the present experiments a reflex inhibition in the intact animal has no significance except in those experiments, in which the respiratory activity decreased in direct connection with the injections, *i. e.* after carbonate injections. Also in the "presso-denervated" animals a contamination in the respiratory reactions from alterations in blood perfusion of the centre or carotid bodies had presumably very little significance, since usually the average blood pressure and the arterial oxygen saturation were high.

Several interesting observations as to the effect of acid and alkali injections upon the arterial blood pressure were made, which will be described and discussed in subsequent papers. In the present work only such blood pressure effects will be mentioned, which may interfere significantly with the respiratory activity and thus are of importance for the interpretation of the respiratory reactions.

### 3. Results and Discussion.

#### **The Response of the Chemoceptively Denervated Centre to Changes in the Arterial $p\text{CO}_2$ and $c\text{H}$ .**

When trying to reveal the relative effects of increased arterial  $p\text{CO}_2$  and  $c\text{H}$  on the activity of the chemoceptively denervated centre it seems appropriate to compare the effects of inhalation of carbon dioxide and intravenous injections of acids. It is recognized that both procedures will eventually evoke hyperventilation. However, inhalation of carbon dioxide gives rise to hypercapnia as well as increased arterial  $c\text{H}$  with substantially unchanged buffering capacity of the blood (normo-carbia), whereas intravenous injection of acids will primarily cause a shift to the acid side by virtue of decreasing the bicarbonate concentration (hypocarbia) and increasing the  $p\text{CO}_2$  of the blood, which will have to pass the lungs on its way to the centre. As will be shown and discussed below, in the case of acid injections the further courses of arterial  $c\text{H}$  and  $p\text{CO}_2$  deviations are not parallel as in hypercapnic hyperventilation, but are greatly influenced by the washing out of carbon dioxide, that takes place in the lungs. It is apparent, that in the type of hyperventilation set up by acid injections, the stimulating factor should be sought among the same variables, that are involved in the true hypercapnic hyperventilation. The important point is, however, that these factors, viz. the arterial  $p\text{CO}_2$ ,  $c\text{H}$  and bicarbonate concentration, do not move in the same directions during hyperventilation of the truly hypercapnic and acid types. By comparing the nature of the respiratory response in the two cases with the behaviour



of the afore-mentioned three variables, it should therefore be possible to draw certain conclusions as to the mode of action of these variables in the arterial blood.

a) *The lacking Parallelism between Pulmonary Ventilation and Arterial  $p\text{CO}_2$  (cH) in Hypercapnic Hyperventilation.*

It is conceivable that hypercapnic hyperventilation in animals, which have been completely deprived of their peripheral chemoreceptor mechanism, should be caused by some stimulating factor, carried by the arterial blood to the centre. The conceptions, that the stimulating factor is either the increase in the arterial cH or  $p\text{CO}_2$  *per se*, require that the pulmonary (alveolar) ventilation should change in close time-correlation to the change in arterial cH or  $p\text{CO}_2$ , when gas mixtures of  $\text{CO}_2$  in  $\text{O}_2$  are temporarily given for inhalation instead of pure oxygen. That the required correlation does not exist became, however, apparent from the recordings of arterial pH, alveolar  $p\text{CO}_2$  and pulmonary ventilation under this type of hypercapnic hyperventilation (fig. 6 A). A sudden increase to a constant and tolerated degree of the  $\text{CO}_2$  concentration in the inspired gas gave rise to a fairly rapid increase in the arterial cH and alveolar  $p\text{CO}_2$ , whereas the pulmonary ventilation increased only gradually, and attained its maximum first after the  $\text{CO}_2$  mixture had been breathed for some minutes. When at the maximum of the hypercapnic hyperventilation pure oxygen was substituted for the  $\text{CO}_2$  mixture, the pulmonary ventilation only gradually decreased to its pre-hypercapnic value. The arterial cH as well as the alveolar  $p\text{CO}_2$  decreased rapidly not only to pre-hypercapnic levels, but actually showed a significant "overshooting" to alkalosis and hypocapnia. After some minutes the three variables had returned to the pre-hypercapnic pattern (fig. 6 A). The lacking parallelism between pulmonary ventilation and arterial cH and  $p\text{CO}_2$  during the acutely induced hypercapnic hyperventilation apparently means that under this condition the activity of the centre is not directly regulated by the arterial cH or  $p\text{CO}_2$ .

It seems reasonable to assume that the respiratory activity, as expressed by the volume of the pulmonary ventilation, would

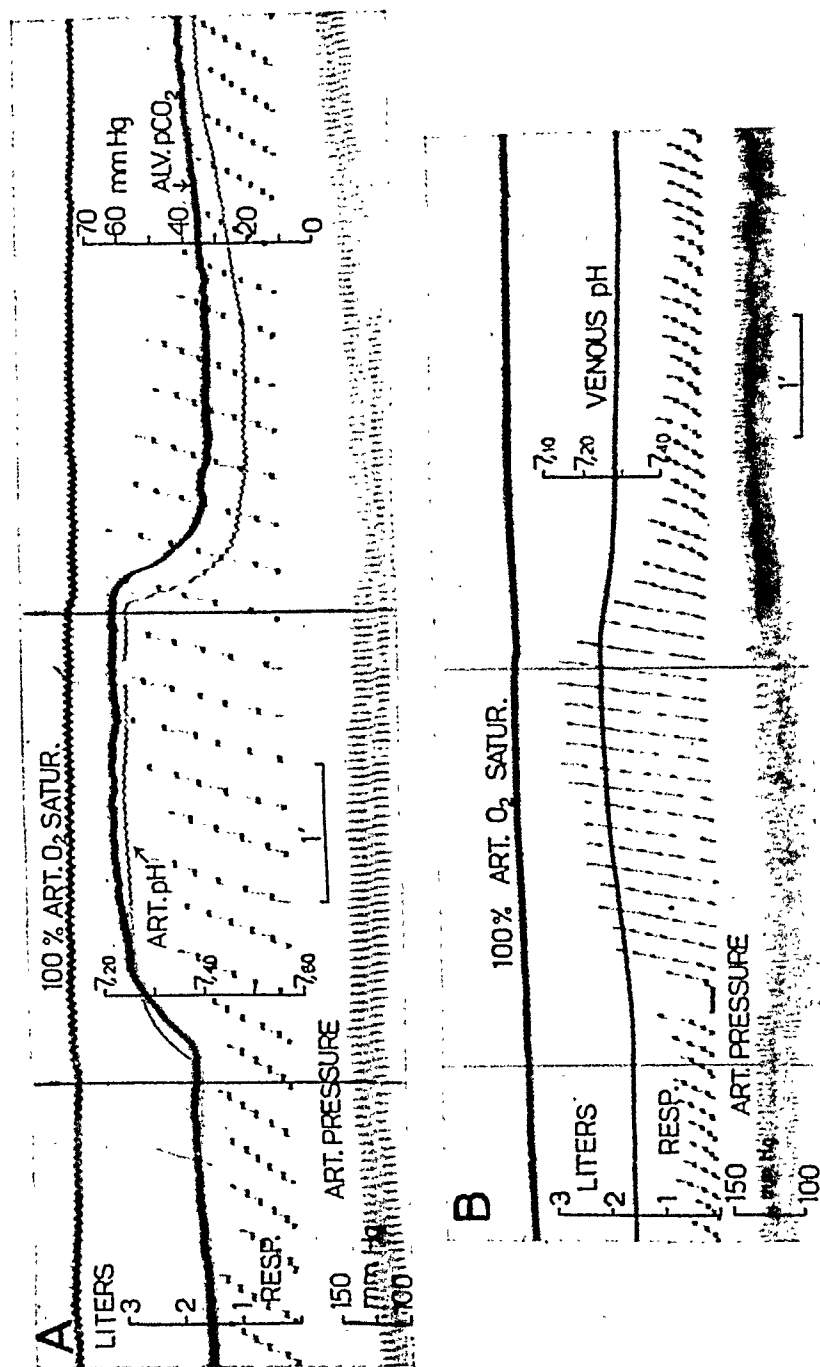


Fig. 6. Response of the chemoreptively denervated centre to a sudden increase of the  $\text{CO}_2$  concentration in the inspired gas. A. Changes of the respiratory activity lagging behind the changes in arterial cH and alveolar  $\text{pCO}_2$ .

B. Changes in pH smaller in the venous than in the arterial blood. Note the closer parallelism between pulmonary ventilation and pH fluctuations in this chart in comparison with A. See text p. 43.

Between markings 6.5 %  $\text{CO}_2$  in  $\text{O}_2$  substituted for  $\text{O}_2$ .

Spikes in the pH-tracing in A: Interruption of the recording for calibration.

substantially be in proportion to the concentration of the chemical stimulus, other factors remaining constant. Hence, in the related experiments the chemical stimulation at or in the centre would be in proportion to the ventilation. It is well known (cf. HENDERSON & HAGGARD 1943) that a certain time is required for a tissue-region to become physically saturated with a gas, which is inhaled at a given pressure. Respiration and circulation remaining constant, the saturation time is dependent mainly on the blood perfusion of the tissue and the solubility coefficient of the gas for the particular tissue. The same holds true for the process of desaturation. From this fact it is obvious that, when the arterial blood is rapidly loaded with  $\text{CO}_2$ , the cells and the extracellular spaces in the respiratory centre will only gradually become saturated with  $\text{CO}_2$  of the same pressure as in the blood. When on the other hand the arterial  $\text{pCO}_2$  is rapidly decreased again, the  $\text{pCO}_2$  of the centre will decrease only gradually. This implies that the changes in  $\text{pCO}_2$  and thus also in molecular  $\text{CO}_2$  concentration and  $\text{cH}$  of the centre lag behind those of the arterial blood. The lacking parallelism between the response of the centre and changes in arterial  $\text{pCO}_2$  and  $\text{cH}$  may therefore be explained, granting that the activity of the centre follows the "intracentral" changes in the molecular  $\text{CO}_2$  concentration or  $\text{cH}$ .

Additional evidence was obtained from the following experiments, illustrated in fig. 11 A. When hypoxic hyperventilation was set up by having the chemoceptively intact animals breathe 8—10 %  $\text{O}_2$  in  $\text{N}_2$  for some minutes, shifting to pure oxygen always caused a marked reduction in ventilation, in most cases even to apnea (cf. also fig. 4 C). The cause of the apnea seems to be, that in the hypocapnic and alkalotic condition, which is produced by hypoxic hyperventilation, the chemosensitive cells of the centre itself become apneic from missing stimulation. Hence, when the hypoxic stimulation of the peripheral chemoreceptors is suddenly removed by breathing oxygen, there will be no respiratory drive at all (see BJURSTEDT 1946). In the present experiments it was demonstrated that the arterial  $\text{cH}$  increased rapidly during the apnea to overshoot its pre-hypoxic level considerably, before respiration started again. Only after a considerable period the ventilation and arterial  $\text{cH}$  had

returned to their pre-hypoxic values. Also in many cases a short apnea was produced by shifting to 6.5 %  $\text{CO}_2$  in  $\text{O}_2$  after the preceding hypoxic hyperventilation, despite the fact that in these cases the arterial blood temporarily became markedly acid. The apneic condition of the centre as opposed to increased arterial  $\text{cH}$  ( $\text{pCO}_2$ ) apparently demonstrates that the centre is not directly stimulated by the arterial  $\text{cH}$  or  $\text{pCO}_2$ . The temporary "overshooting" of the arterial  $\text{cH}$ , on the other hand, seems to establish that the actual stimulus was only slowly built up. Granting the activity of the centre follows its own  $\text{cH}$  or  $\text{pCO}_2$ , the slow increase of the stimulation supports the opinion, that the centre was only gradually loaded with  $\text{CO}_2$  from the blood. This conception seems to be inconsistent with the original intracellular acidity theory of GESELL, advanced in 1923 (cf. GESELL 1925), according to which the activity of the respiratory centre is fundamentally a function of its own intracellular acidity by virtue of its own acid metabolism. In the experiments described above, the central metabolic stimulation by endogenous acid was in any case very slight, compared to the effect of exogenous carbon dioxide. Otherwise, the pulmonary ventilation would have started again much sooner, which would have prevented the strong acid shift in the arterial blood reaction.

If the opinion is correct, that the changes in  $\text{pCO}_2$  of the centre lag behind those of the arterial blood in consequence of saturation and desaturation processes, the changes in  $\text{pCO}_2$  and  $\text{cH}$  of the venous blood coming from the centre would be smaller than those of the arterial blood. In order to obtain evidence on this point, the  $\text{pH}$  of the blood in the internal jugular vein was recorded continuously in experiments on three dogs. When a gas mixture of 6.5 %  $\text{CO}_2$  in  $\text{O}_2$  was temporarily given for inhalation instead of  $\text{O}_2$ , it was observed that the venous  $\text{cH}$  increased only very gradually, and to a much lesser degree, than did usually the arterial  $\text{cH}$  (cf. fig. 6A and 6B). On readministration of  $\text{O}_2$ , the  $\text{cH}$  continued to increase for a short period before returning fairly slowly to its pre-hypercapnic value (fig. 6B). Also, only less pronounced changes in venous  $\text{pH}$  were observed when  $\text{O}_2$  was administered after hypoxic hyperventilation. Though the  $\text{pH}$  was measured in the mixed

venous blood coming from the head, it seems probable that the recordings closely reflected the reaction of the blood returning from the centre. When comparing the respiratory response to  $\text{CO}_2$  with arterial and venous  $\text{cH}$ , it becomes obvious that the parallelism is closer in respect to venous  $\text{cH}$ . Actually it may be possible to correlate the response to a  $\text{cH}$  ( $\text{pCO}_2$ ), the value of which lies somewhere between the arterial and the venous  $\text{cH}$  ( $\text{pCO}_2$ ). These observations seem to speak in favour of the conception, that the centre only gradually comes into  $\text{CO}_2$  equilibrium with the blood during its passage through the centre tissue.

Several possibilities may exist as to the mode in which the  $\text{cH}$  or  $\text{CO}_2$  acts upon the centre, *e. g.* 1) by directly affecting or stimulating the surface of chemosensitive cells of the centre, 2) by exaggerating the excitability of the centre by attacking its synaptic interconnections, or 3) by a specific stimulating effect only after having entered the interior of the cells.

Summing up the above results the following conclusions may be drawn. The lack of time-correlation of arterial  $\text{pCO}_2$  or  $\text{cH}$  and pulmonary ventilation shows that the activity of the centre is not directly regulated by the arterial  $\text{pCO}_2$  or  $\text{cH}$ . The lacking parallelism may, however, be accounted for, granting the  $\text{cH}$  or molecular  $\text{CO}_2$  concentration within the centre is the actual stimulus. With a certain time lag either of the two variables is altered in relation to changes in arterial  $\text{pCO}_2$ , the time lag being due to rather slow processes of saturation and desaturation in the centre in respect to  $\text{CO}_2$ . On the other hand, the data from these experiments give no information as to the question whether the  $\text{cH}$  or  $\text{CO}_2$  *per se* of the centre is the actual stimulus, since they varied here simultaneously and in the same direction.

#### b) *The Effects of Deviations from the Normocarbic Condition.*

The main arguments against the first "reaction theory" of WINTERSTEIN (cf. p. 12), according to which the respiratory activity is regulated by the  $\text{cH}$  of the blood, were the lack of parallelism of breathing and arterial  $\text{cH}$  during oxygen deficiency and following intravenous injection of  $\text{NaHCO}_3$ .

However, after the discovery of the peripheral chemoreceptor mechanism, the first condition seems no longer to be contradictory to the theory, if this is restricted to refer only to the centre itself. Actually, the self-engendered activity of the centre decreases in close relationship to the decreasing arterial cH during acute and tolerated oxygen deficiency, as has been clearly demonstrated by BJURSTEDT (1946). Whereas observations of lacking parallelism between breathing and blood cH following  $\text{NaHCO}_3$  injection in the chemoceptively intact animal are numerous, only incomplete information on this point after complete chemoceptive denervation seems to be available in the literature. According to GESELL (1939), an increased ventilation arises from injection of  $\text{NaHCO}_3$  in the denervated animal, but no direct data as to the blood cH and  $\text{pCO}_2$  were given. NIELSEN (cf. p. 15) ascribes the increased respiratory activity during ammonium chloride acidosis to an increased excitability of the centre to  $\text{CO}_2$ , but gives no clue to how such an increased excitability to  $\text{CO}_2$  during acidosis should be explained, apart from an implication, that it might be caused, directly or indirectly, by the changes in blood reaction. The suggestion of SCHMIDT (1941) that the increased excitability of the centre during acidosis may partly or even entirely be due to the reflexes from the aortic and carotid bodies seems not to have been experimentally confirmed.

From these considerations it is obvious, that reliable information on the inherent mechanism of stimulation of the centre itself can hardly be obtained from experiments on animals with active chemoreflex drive. More significant data in this respect would be obtained after freeing the centre from chemoreceptor connections. By observing continuously the alterations in pulmonary ventilation, arterial pH and  $\text{pCO}_2$  associated with experimentally evoked variations in the buffering system, it should be possible to draw certain conclusions as to the nature of the activating stimulus within the centre.

It was mentioned above that the hemo-respiratory reactions following administration of acid and alkali varied considerably according to the rate of injection. The effects of rapid intravenous injections of relatively small doses of acids and alkalis in the presso- and chemoceptively denervated dog are shown in

fig. 7A and B. The predominant reactions following the injections of HCl and  $\text{Na}_2\text{CO}_3$  seem to harmonize with those theories, according to which the activity of the centre is regulated by the arterial cH or  $\text{pCO}_2$ , as well as with the conceptions that the  $\text{CO}_2$  or the cH of the centre itself is the dominating chemical stimulus. However, a more close examination of the recordings reveals that the changes in respiratory activity lag behind those in arterial cH and  $\text{pCO}_2$ . This fact yields further support to the conception that the centre is not directly stimulated by the arterial cH or  $\text{pCO}_2$ , which becomes still more apparent from the effects produced by injection of  $\text{NaHCO}_3$ . The question then arises, whether the  $\text{CO}_2$  or the cH in the centre is the dominant factor in the central chemical control of breathing.

The lack of time-correlation between changes in arterial  $\text{pCO}_2$  and ventilation seems to be in harmony with both the theory of a specific action of  $\text{CO}_2$  and the acidity theory, remembering the fact shown above, that the changes in  $\text{pCO}_2$  of the centre lag behind those of the arterial blood. The conditions of an initially increased arterial  $\text{pCO}_2$  and ventilation following injections of HCl and  $\text{NaHCO}_3$  as well as of a temporary reduction in arterial  $\text{pCO}_2$  and ventilation after  $\text{Na}_2\text{CO}_3$  may be explained by either of the theories, since both the molecular  $\text{CO}_2$  concentration and cH of the centre vary in proportion to its  $\text{pCO}_2$ . The further course of the hemorespiratory reactions is, however, contradictory to the theory of a specific action of  $\text{CO}_2$ . Thus the period of decreased arterial  $\text{pCO}_2$  after  $\text{Na}_2\text{CO}_3$  injection is followed by a condition of increased  $\text{pCO}_2$  and subnormal ventilation (fig. 7B), whereas some minutes after the injection of HCl the arterial  $\text{pCO}_2$  is lower and the ventilation larger than before this injection. These deviations in the arterial  $\text{pCO}_2$  cannot to any significant degree be due to alterations in the  $\text{CO}_2$  production, since they appeared within the course of some minutes, but must mainly be due to actual changes in alveolar ventilation. Moreover, there seems to be no reason to believe that, during these later periods, the  $\text{pCO}_2$  of the centre differed significantly from that of the blood, for the arterial  $\text{pCO}_2$  was now fairly constant. It follows then that the molecular  $\text{CO}_2$  concentration of the centre during the periods mentioned was altered in opposite direction

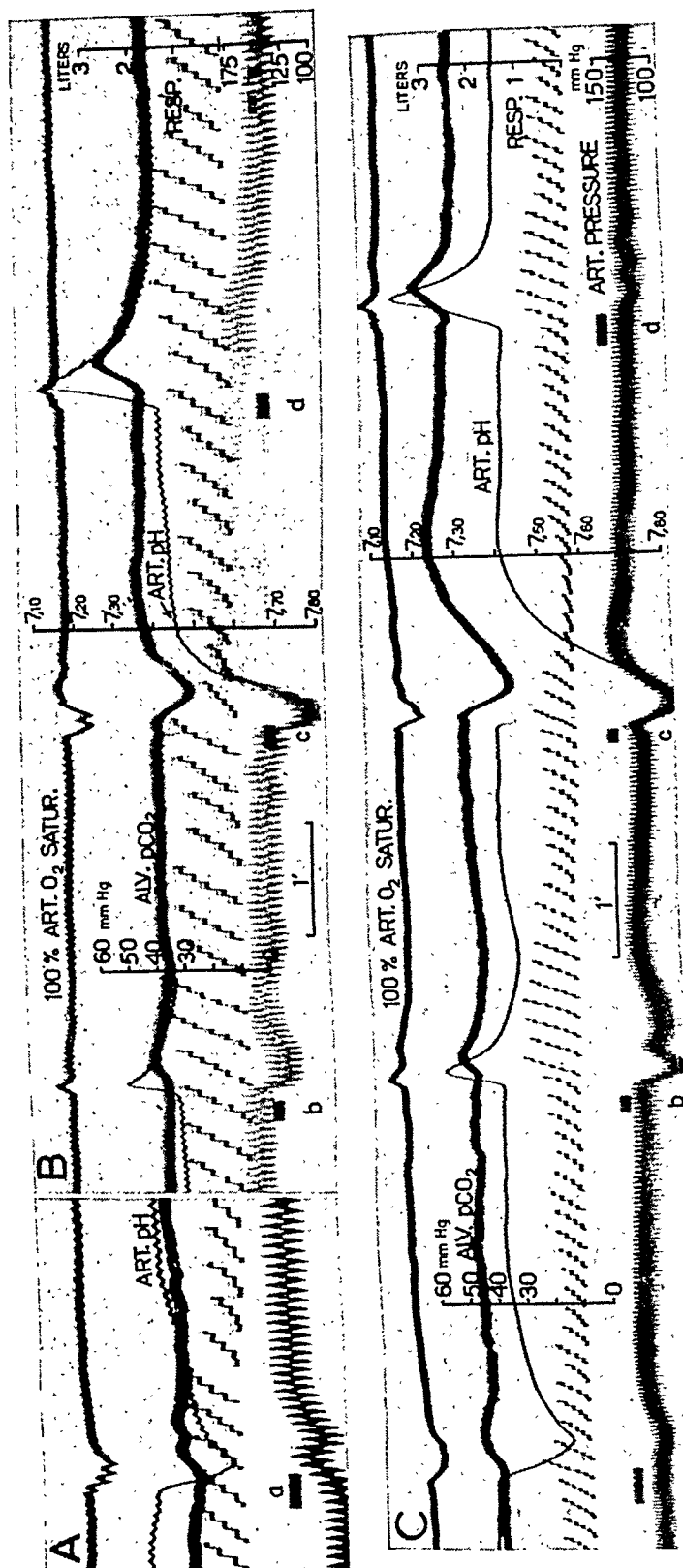


Fig. 7. Effects of rapid, intravenous injections of acid and alkaline solutions after (A and B) and before (C) section of the vagi and sinus nerves. The respiratory response is slower and lesser in A and B than in C. Note the marked increase in ventilation after injection of CH<sub>3</sub>COOH. Further discussion pp. 45—50.

- NaHCO<sub>3</sub>, 1 ml/kg of a 8 per cent solution.
- CH<sub>3</sub>COOH, 0.1 ml/kg of a 2 per cent solution.
- Na<sub>2</sub>CO<sub>3</sub>, 1 ml/kg of a 8 per cent solution.
- HCl, 0.5 ml/kg of a N/2 solution.



to the changes in respiratory activity. Hence, the present experiments do not support the theory of a specific action of  $\text{CO}_2$ , if this theory implies that the effectiveness of  $\text{CO}_2$  as a stimulus is in proportion to its molecular concentration.

The accessory theory proposed by NIELSEN, that the excitability of the centre to  $\text{CO}_2$  may become altered during changes in the blood reaction is of course acceptable, but how such changes are brought about was not explained by NIELSEN. The suggestion of SCHMIDT, that the chemoreflex drive may be responsible for such alterations is obviously not relevant to the experiments described above. However, granting the basal inherent activity of the centre itself is mainly controlled by its own  $\text{cH}$ , the changes in "excitability" of the centre to  $\text{CO}_2$  during acid-base displacements may be explained on a quite logical and definite basis, as pointed out by GESELL (1939). Thus in acidosis, produced by injection of  $\text{HCl}$ , the bicarbonate content of the blood is reduced, which means not only that the carbonic acid/bicarbonate ratio, and hence the blood  $\text{cH}$ , is increased at a given  $\text{pCO}_2$  or a uniform production of metabolic  $\text{CO}_2$ , but also that the excitability of this ratio to a given change of the  $\text{pCO}_2$  is increased. In alkalosis, produced by injection of  $\text{Na}_2\text{CO}_3$ , the reversed condition is prevailing. On the other hand, this argument can be used for the centre itself only in case the buffering alkali of the centre changes in relation to that of the blood, but any direct proof on this point seems not to be available in the literature.

The important question, whether and at which rate acid-base displacements in the blood will be reflected within the centre seems to rest mainly on the permeability characteristics of the cells within the centre. It is well known that  $\text{CO}_2$ , and probably also  $\text{H}_2\text{CO}_3$ , penetrates the cell membrane very rapidly (cf. JACONS 1920). It can therefore be expected, that during acid-base displacements in the blood the  $\text{H}_2\text{CO}_3$  concentration within the centre fairly rapidly becomes substantially the same as in the blood, assuming the solubility coefficient of  $\text{CO}_2$  is similar for blood and tissues. Therefore, the question as to the rate of penetration of the  $\text{H}$ -ion itself is probably of minor importance for the  $\text{H}$ -ion equilibrium between blood and centre, since the  $\text{cH}$  always is determined by the carbonic acid/bicarbonate ratio.

It is generally considered, that strong acids such as HCl only very slowly penetrate the cell membrane. Also this conception seems to be irrelevant to the present experiments, for fixed acids entering the blood will immediately react with the buffering alkali, and thus cannot exist freely in the blood or tissues. Alkalies liberated into the blood stream will combine with the  $\text{CO}_2$  of the blood, and are thus converted into bicarbonates. Accordingly, intravenous injections of acids and alkalies will produce alterations in the buffering alkali content of the centre to the extent in which the cell membranes within the centre are permeable to the anions of acids and to the cations of alkalies. The opinion vigorously maintained for many years, that certain cells may be completely impermeable to certain ions, can no longer be held in the light of evidence, which was based on the new technique with radioactive elements (for reference, see HEILBRUNN 1943). On the other hand, it has also been established that the rate of penetration may differ considerably for different ions, and moreover, that each type of cell shows special characteristics of permeability. The question then arises as to which type the cells within the centre may belong, and as to the rate of penetration of those ions, the equilibrium of which is disturbed during acid-base displacements in the blood.

Returning to the later course of the hemo-respiratory reactions following rapid injection of  $\text{Na}_2\text{CO}_3$  (fig. 7B), the condition of a fairly constant arterial  $\text{pCO}_2$  and of a gradually increasing  $\text{cH}$  denotes that the buffering alkali of the blood decreases gradually. This fact obviously demonstrates that "alkali is driven out" of the blood into the tissues and presumably also into the centre, though nothing can be said whether this is due to an actual migration of alkali into the tissues or to a reversed migration of acid radicals. The main point is, however, that the buffering alkali content of the centre increases. Consequently the denominator in the carbonic acid/bicarbonate fraction, and hence the pH within the centre, increases. The subnormal ventilation prevailing in the steady state after administration of  $\text{Na}_2\text{CO}_3$  may thus be explained, assuming the  $\text{cH}$  within the centre itself to be the dominating regulator of the inherent activity of the centre. The hemo-respiratory reactions

following rapid injection of  $\text{NaHCO}_3$  (fig. 7 A) may be accounted for in the same way. The sudden rise in arterial  $\text{pCO}_2$  is followed by an increase in the  $\text{pCO}_2$  and thus in  $\text{cH}$  of the centre, and hyperventilation is set up. Gradually the buffering alkali content of the centre raises, thereby depressing the  $\text{cH}$ , and consequently the ventilation is again reduced.

If we presume that the blood is suddenly charged with an acid radical, that may rapidly penetrate the membranes of the cells within the centre, the ventilation should probably increase to such a degree that, after the initial rise in arterial  $\text{cH}$ , a temporary alkaline shift in the blood should be produced due to excessive washing out of  $\text{CO}_2$ . In order to confirm this conception it was considered appropriate to make rapid, intravenous injections of acetic acid, since it is well established that weakly dissociated acids have a strong penetrating power. As shown in fig. 7 B the expected effects were actually produced. Thus, although the initial rise in arterial  $\text{cH}$  and  $\text{pCO}_2$  was considerably less than that following injection of  $\text{HCl}$ , the increase in ventilation was the same or even larger in the former case. These findings are evidently in harmony with the opinion, that the activity of the centre is mainly regulated by the  $\text{cH}$  within the centre, thus giving strong support for a theory of intracentral acidity.

As mentioned above the permeability characteristics may vary considerably in different types of cells. It is therefore reasonable, that the rate of ion equilibration following experimental acid-base displacements in the blood should vary considerably in different tissues. This conception is supported by the findings of KATZ & BANUS (1927), that muscle tissue does not contribute significantly to the buffering of  $\text{HCl}$ , liberated into the blood stream. Also, ROUS & BEATTIE (1926) found that the changes in  $\text{cH}$  following injection of fixed acid showed a marked variation in different tissues. From the conclusion of the present work, that the buffering alkali in the centre changes rather rapidly, when acid or alkali is added to the blood, it follows that the ions in view probably pass through the cell membranes within the centre fairly rapidly. This statement does, however, not imply that, when equilibrium is prevailing, there is equality of ion concentration in blood and centre. Thus the

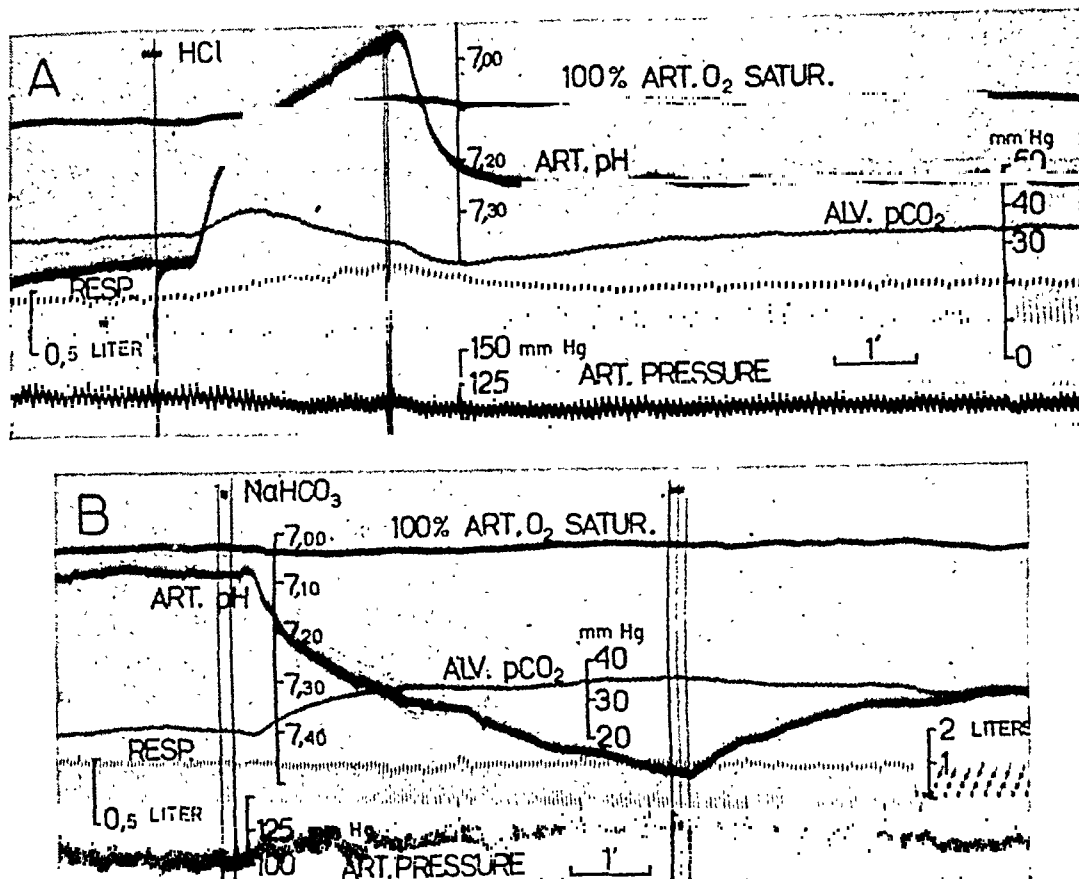


Fig. 8. Hemo-respiratory reactions in the chemoceptively denervated dog following intravenous injections of acid and alkaline solutions at a "moderate" rate.

- A. Between markings injection of N/2 HCl, 2.5 ml/kg. The intracerebral changes in pH in connection with this injection and the correlation between ventilation and intracerebral reactions are discussed on pp. 51—52.
- B. Between markings injection of a molar solution of NaHCO<sub>3</sub>, 4 ml/kg. The intracerebral reactions caused by this injection are discussed in the text and correlated with the changes observed in pulmonary ventilation pp. 52—54.

cH within the centre may differ significantly from the blood cH, even in conditions of steady state.

Assuming therefore, that changes in the buffering alkali within the centre will follow those of the blood in rather close time-correlation, whereas in certain other tissues such equilibration is slower, one would expect that the hemo-respiratory reactions produced by injection of fixed acid at a moderate rate would be as follows (cf. fig. 8 A). During the course of the injection the buffering alkali content of the blood

will rapidly decrease and the  $p\text{CO}_2$  temporarily rise due to liberation of  $\text{CO}_2$  from the bicarbonates. At the same time the  $\text{cH}$  within the centre will increase as a result of the rise in  $p\text{CO}_2$  and the diminishing buffering alkali content within the centre. Thus hyperventilation is set up, thereby causing a reduction in the alveolar  $p\text{CO}_2$ . After the injection is completed, the bicarbonate content of the blood gradually increases again as alkali is called into the blood from tissues with low permeability. As a consequence the buffering capacity of the centre now increases, and thus the hyperventilation is reduced. Finally a state of equilibrium is attained, where the carbonic acid/bicarbonate ratio, and hence the  $\text{cH}$ , within the centre is somewhat increased, and consequently also the alveolar ventilation. It was also found that the more slowly the injections were made, the less was the initial rise in arterial  $p\text{CO}_2$ . This observation may be accounted for as due to the more gradual liberation of  $\text{CO}_2$  from bicarbonates in these cases, and furthermore to the increased ventilation set up mainly due to the decreasing bicarbonate content in the centre, the latter process also meaning a locally increased  $\text{cH}$ .

During the course of injection of  $\text{NaHCO}_3$ , made at a moderate rate, both the arterial  $p\text{CO}_2$  and  $\text{pH}$  gradually increased, whereas the ventilation remained unchanged or decreased a little (fig. 8 B). According to an intracentral acidity theory the  $\text{cH}$  within the centre should therefore remain unchanged or be slightly depressed. As mentioned above it is highly probable that the buffering alkali in the centre follows the changes of that in the blood in rather close time-correlation, but this does not mean, that the changes in concentration of bicarbonates are the same in blood and centre. Thus, although the carbonic acid/bicarbonate ratio in the arterial blood diminished considerably, this ratio may have decreased only insignificantly within the centre. Hence, only insignificant changes in ventilation appeared. The gradual reduction in the bicarbonate concentration of the blood, and thus of the centre, after the injection was completed (see fig. 8 B), was probably due to migration of alkali into tissues with low permeability. Simultaneously the  $p\text{CO}_2$  of the blood and the centre diminished, thereby preventing a rise in or even depressing the  $\text{cH}$  of the

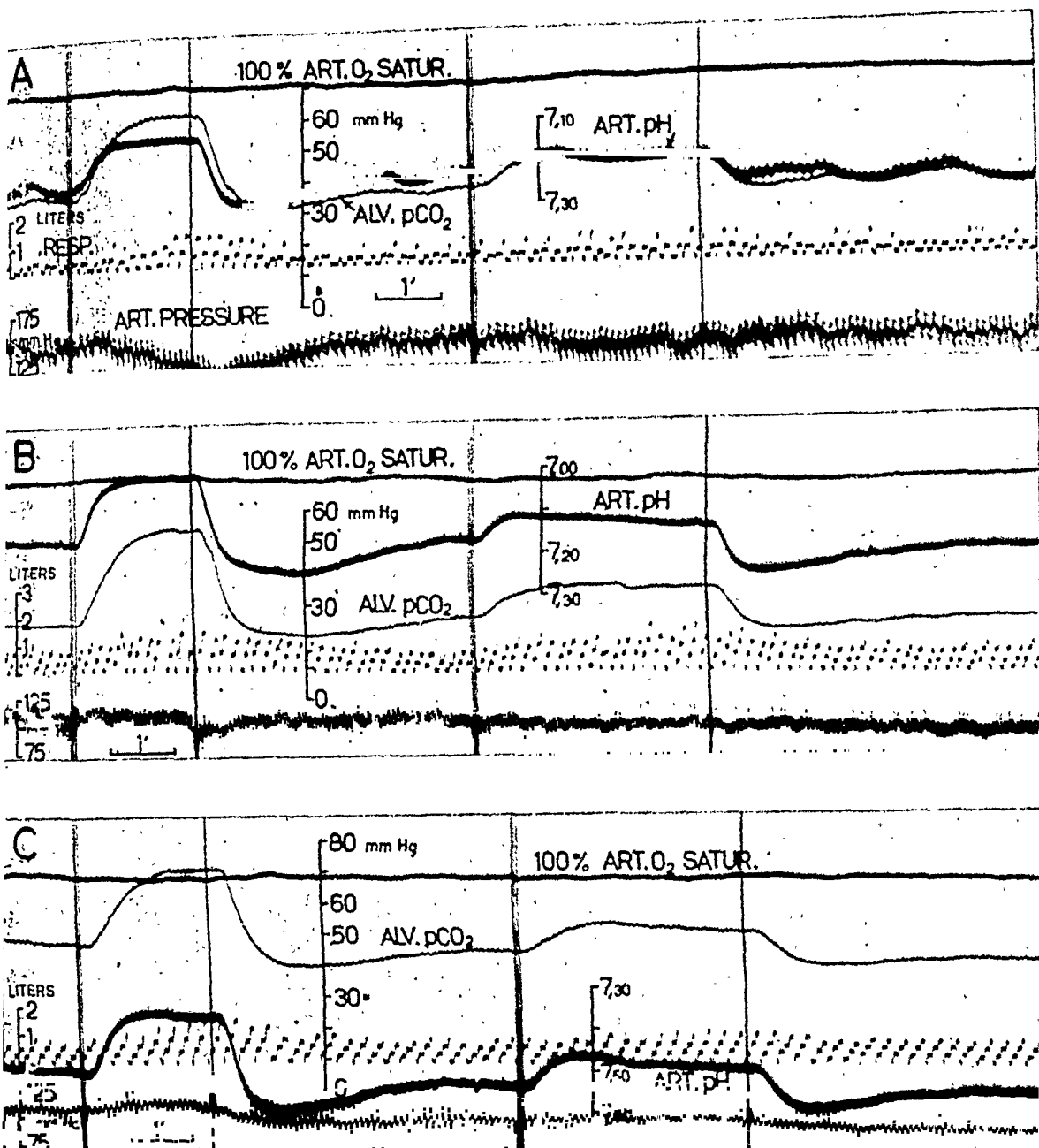


Fig. 9. Same dog as in figure 4. Influence of deviations from the normocarbic condition upon the activity of the chemoceptively denervated centre.

A. Normocarbic condition. — B. Hypocarbic condition ("metabolic acidosis"). — C. Hypercarbic condition ("metabolic alkalosis").

The respiratory activity is greater in hypocarbia and smaller in hypercarbia than in the normocarbic condition. The respiratory response to an increase of the CO<sub>2</sub> concentration of the inspired gas is also greater in hypocarbia and smaller in hypercarbia, if compared with the response under normocarbica.

Between first two markings: Sudden change from O<sub>2</sub> to 6.5 % CO<sub>2</sub> in O<sub>2</sub>.

Between following two markings: Sudden change from O<sub>2</sub> to 2.8 % CO<sub>2</sub> in O<sub>2</sub>.

centre. Thus the slight reduction in ventilation after the completion of the injection is rendered intelligible.

The increased response of the respiratory apparatus to  $\text{CO}_2$  during acidosis obviously cannot be entirely due to chemoreflex influence, when considering that the response of the completely chemoceptively denervated centre to  $\text{CO}_2$  is increased during acidosis, produced by fixed acids. The above-mentioned experiments with rather slow injections of  $\text{HCl}$  have already clearly demonstrated that in the denervated dog hyperventilation can be produced in spite of reduced arterial  $\text{pCO}_2$ . However, it was considered appropriate to make another series of experiments, illustrated in fig. 9. The hemo-respiratory reactions following a sudden increase in the  $\text{CO}_2$  concentration of the inspired gas were here observed in the normocarbic (fig. 9 A), hypocarbic (fig. 9 B), and hypercarbic (fig. 9 C) condition. Hypocarbica was produced by injections of large doses of  $\text{HCl}$ , whereas hypercarbica was induced by administering large doses of  $\text{NaHCO}_3$ . The recordings were postponed until a steady condition in respect to pulmonary ventilation, alveolar  $\text{pCO}_2$  and arterial  $\text{pH}$  was established. If comparing the three recordings it becomes evident that, under oxygen inhalation, the activity of the centre itself, as expressed by the volume of alveolar ventilation (alveolar  $\text{pCO}_2$ ), is greater in acidosis, and less in alkalosis than in normocarbic condition. The conclusion is obviously that the response of the centre to  $\text{CO}_2$  can change without active chemoreflex drive. The observations are in agreement with an intracentral theory, according to which the changes in excitability are due to alterations in the value for the denominator of the carbonic acid/bicarbonate ratio within the centre, which causes changes in the "excitability" of this ratio to a uniform production of metabolic  $\text{CO}_2$ . In hypocarbica the ratio, and hence the  $\text{cH}$ , is increased, and hyperventilation is set up, whereas in hypercarbica the ratio, and thus the  $\text{cH}$ , is depressed, which leads to reduced ventilation.

According to the theory, a given rise in alveolar  $\text{pCO}_2$  should produce a greater increase in ventilation during hypocarbica than during hypercarbica. This statement was confirmed in the mentioned experiments, for during the periods of inhalation of  $\text{CO}_2$  gas mixtures, the ventilation increased considerably more

in the acidotic than in the alkalotic condition, though the rises in alveolar  $p\text{CO}_2$  were substantially of the same order (cf. fig. 9 B and 9 C). The rather surprising observation that the increase in arterial cH was much the same in the two conditions must not involve similarity in rise of intracentral cH. It follows from the  $\text{CO}_2$  dissociation curves of the blood that, in hypocarbic conditions, the bicarbonate concentration increases relatively more than under hypercarbia after a given rise in  $p\text{CO}_2$ , which would be the main cause of the similarity in rise of arterial cH. Since hemoglobin is the main source of the liberated alkali, it seems probable that the bicarbonate concentration within the centre should increase significantly less than in the blood. Hence, assuming similar solubility coefficient of  $\text{CO}_2$ , the changes in intracentral cH would be greater than those in the blood, and also relatively greater in hypocarbic than in hypercarbic conditions.

#### **Interaction of Centrogenic and Chemoreflex Drives during Acid-Base Displacements in the Blood.**

As pointed out in the definition of the problem very little is known concerning the relative importance of central and chemoreflex components during conditions of metabolic disturbances in the acid-base balance of the blood. Opinions differ as to the rôle of the chemoreflex drive during normocarbic conditions in combination with high arterial oxygen saturation and varying alveolar  $p\text{CO}_2$ .

It was also pointed out that the most direct information as to the interaction of the two components at a given situation will be obtained from experiments, in which the chemoreflex component is suddenly and temporarily withdrawn by cold-blocking the chemosensory signals. However, during very rapid and short-lasting changes in the acid-base balance, *e. g.* after rapid injection of acid and alkaline solutions, the cold-blocking technique is not suitable for this purpose. In these situations a comparison of the hemo-respiratory reactions of the chemoceptively intact animal with those of the chemoceptively denervated animal, however, yielded some information as to the rôle of the chemoreflexes.



a) *Hypercapnic Hyperventilation.*

The fact, which was confirmed in above-mentioned experiments, that the centre itself responds to increased  $\text{CO}_2$  in the inspired gas makes it evident, that the importance of the chemoreflex drive in the hypercapnic hyperventilation is not so conspicuous as in the hypoxic hyperventilation. It is well established that, during acute progressive hypoxemia, the chemoreflex component gains the upper hand of the central component, and furthermore that under eupneic conditions the peripheral chemoreceptors are moderately active. It is also known that both  $\text{CO}_2$  and "normal  $\text{O}_2$ -want" in the arterial blood are factors in maintaining electrical discharge from the chemoreceptors during eupnea. Whereas it now seems generally accepted that part of the total respiratory activity during eupnea is maintained by a tonic influence from hypoxic stimulation of the peripheral chemoreceptors, opinions still differ as to the importance of reflex stimulation by the arterial  $\text{pCO}_2$  during eupnea.

Before the chemoreflex component in the hypercapnic hyperventilation will be discussed, it therefore seems appropriate to inquire into the quantitative significance of this component in the respiratory activity during 100 per cent arterial oxygen saturation and normal  $\text{pCO}_2$ . The respiratory effects of a sudden withdrawal of the chemoreflex drive during condition of oxygen inhalation should give the most direct information as to the rôle of the chemoreflex component for the maintenance of normal  $\text{pCO}_2$ . As shown in fig. 10 a temporary chemoreflex disconnection of the centre results in a small reduction in the alveolar ventilation, indicating that the centre alone is unable to maintain the  $\text{pCO}_2$  at the usual level. The conclusion is that the arterial  $\text{pCO}_2$  even under high arterial oxygen saturation stimulates respiration also over the chemoreflex mechanism, and that, consequently, the chemoreflex component should be a necessary factor in the finer adjustment of breathing under physiological conditions with respect to carbon dioxide. Hence, the condition is that of a chemoreflex pace-making, as is also the case with respect to the maintenance of normal arterial oxygen. The possible complication of an indirect stimulating

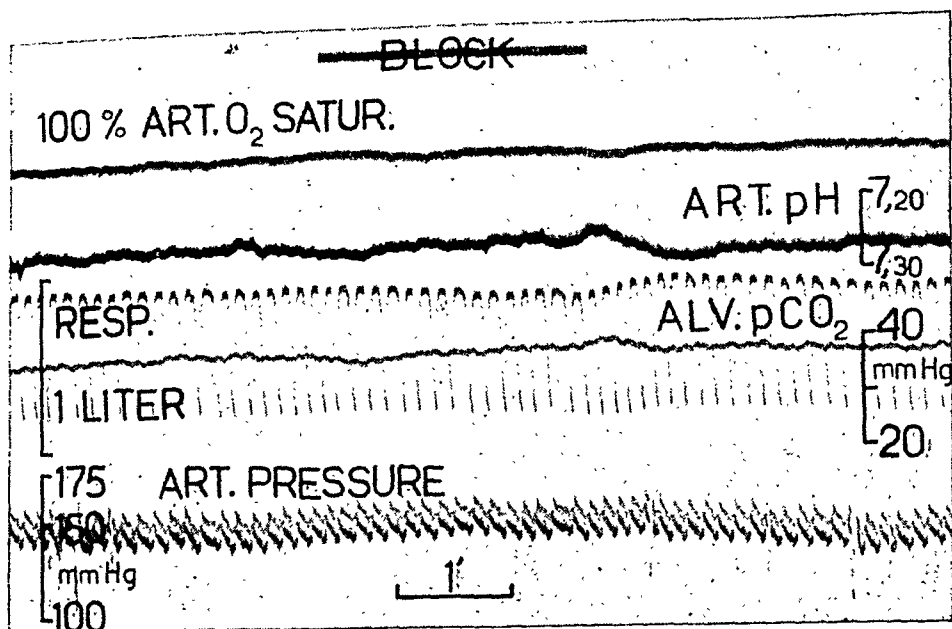


Fig. 10. Effects of withdrawal of the chemoreflex component during oxygen breathing. The decrease of the pulmonary ventilation is small but certain, as revealed by the increase in arterial cH and alveolar  $p\text{CO}_2$ . This indicates that even under conditions of high arterial  $\text{O}_2$  saturation the chemoreflex mechanism has a tonic influence on the respiratory activity. See pp. 56—57.

effect on “central” respiration by increased metabolism after section of the sinus and depressor nerves is presumably of minor importance in the present short-lasting cold-blocking experiments (cf. EULER & LILJESTRAND 1935, HAHN 1940). As to the tonic reflex inhibition on the respiratory activity from the pressoreceptors, this complication is apparently not relevant to the present experiments, since the pressosensory fibres had been sectioned in advance. The slight rise in the blood pressure during the blocking period cannot to any significant degree be responsible for the decrease in ventilation.

Also the following observations are in harmony with the conception that, under condition of oxygen inhalation, section of the sinus nerves results in decreased ventilation and increased alveolar  $p\text{CO}_2$ , and that the main cause of these effects is the withdrawal of a reflexly stimulating effect of  $\text{CO}_2$  upon respiration over the glomus mechanism (cf. EULER & LILJESTRAND 1936, 1940). Thus after vagotomy, section of the sinus nerves under oxygen breathing resulted in decreased alveolar ventilation (increased alveolar  $p\text{CO}_2$ ), whereas the arterial cH

increased (cf. fig. 4 B and 9 A). The withdrawal of the respiratory inhibitory action of the pressoreceptors could only produce a counteracting effect, thereby to a certain degree masking the full effect of chemoceptive denervation. The possible influence of the moderate rise in arterial blood pressure on respiration was probably of less importance in these experiments, since the arterial oxygen saturation was kept at 100 per cent.

It may be anticipated that, since the activity of the chemoreceptors are known to increase with increasing arterial  $p\text{CO}_2$  (EULER, LILJESTRAND & ZOTTERMAN 1939), also the chemoreflex drive should increase under these conditions. In consequence one would expect that repeated, temporary withdrawals of the chemoreflex drive during the course of progressive hypercapnia should cause an increasing absolute reduction in the hypercapnic hyperventilation. However, as already discussed (p. 18), "chemoreceptor excitation" is a thing quite different from "chemoreflex drive". Actually, direct experimental evidence did not confirm the above-mentioned anticipation. As shown in fig. 11 B, temporary cold-blocking of the peripheral chemosensory impulses causes a small reduction in the alveolar ventilation only at the beginning of progressive hypercapnia, induced by having the animal breathe in a respirometer without  $\text{CO}_2$  absorption and containing pure oxygen. In all experiments of this type (rebreathing with  $\text{CO}_2$  accumulation) no block effect could be observed as soon as the alveolar  $p\text{CO}_2$  exceeded about 60 mm Hg.

From experiments, in which the alveolar  $p\text{CO}_2$  was kept higher than normal, but constant, by means of inhalation of constant mixtures of  $\text{CO}_2$  in  $\text{O}_2$  it also became clear, that blocking the chemoreflexes does not influence the amount of hyperventilation, when the alveolar  $p\text{CO}_2$  exceeds about 60 mm Hg.

These findings are in sharp contrast to cold-blocking experiments during acute progressive hypoxemia or during a constant, acute and tolerated degree of oxygen deficiency. In the latter cases the withdrawal of the chemoreflex drive causes marked reduction in ventilation, as shown by GESELL, LAPIDES & LEVIN (1940) and BJURSTEDT (1946). These observations have been confirmed in the present work, exemplified in fig. 11 A. Thus

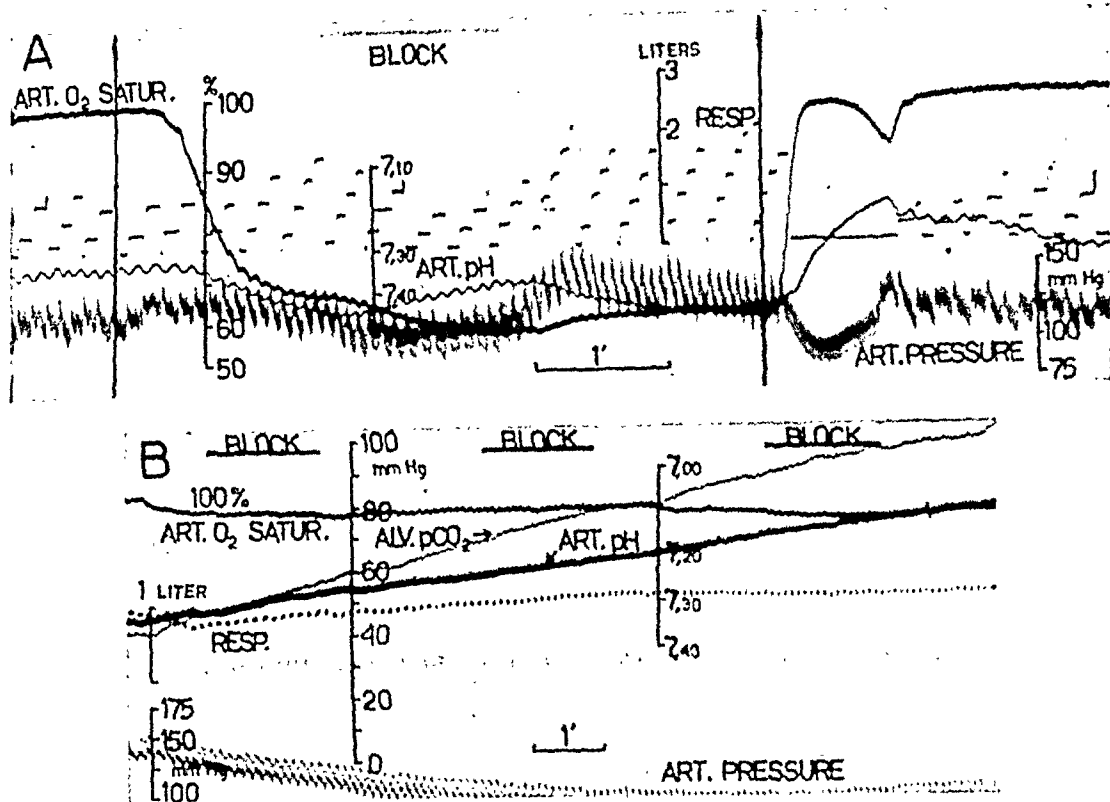


Fig. 11. A. The figure demonstrates the important rôle of the chemoreflex component during acute hypoxic hyperventilation, revealed by the marked decrease of the ventilation when the chemosensory impulses are blocked. Note the long-lasting apnea when oxygen is again administered. The arterial pH increases temporarily above its pre-hypoxic level before the respiration starts again, indicating that the arterial pH does not directly regulate the respiratory activity. Between vertical markings 8.2 % O<sub>2</sub> in N<sub>2</sub> substituted for O<sub>2</sub>.

B. Repeated withdrawals of the chemoreflex drive during progressive hypercapnic hyperventilation. The figure shows that the chemoreflex component has only a minor share in the respiratory activity during moderate degrees of hypercapnia, the hyperventilation of high grade hypercapnia being entirely centrogenic (p. 58).

the remarkable fact is revealed that, when a progressively increasing hyperventilation is set up by increasing hypoxic stimulation, the chemoreflex component gradually gains the upper hand of the central component, but when caused by progressive hypercapnia the chemoreflex component in the hyperventilation gradually decreases. The situation becomes interesting, when remembering that in both types of hyperventilation the activity of the peripheral chemosensitive cells is known to increase gradually. It is therefore evident, that the outcome of the chemoreflex signals, i.e. the chemoreflex drive,

is modified by some intermediary mechanism. It seems reasonable to assume, that the cause of this modifying influence should be sought among those variables, which differ most conspicuously in the hypoxic and the hypercapnic types of hyperventilation, viz. the arterial  $p\text{CO}_2$ ,  $\text{cH}$  and  $p\text{O}_2$ . GESELL, LAPIDES & LEVIN concluded from their experiments, earlier referred to, that increasing  $p\text{CO}_2$  exerts an increasing central blocking action on the signals, which are set up in the chemoreceptors, and conversely, that decreasing  $p\text{CO}_2$  diminishes this central blocking action of  $\text{CO}_2$ , thereby potentiating the signals arising in the chemoreceptors. Direct evidence for this hypothesis was, however, not presented. BJURSTEDT (1946) presented evidence, that the increase of the chemoreflex drive during the development of the respiratory alkalosis under acute oxygen deficiency is not only determined by the hypoxemic stimulus to the chemoreceptors, but is actually potentiated by the alkaline change per se.

Whether the increased  $p\text{CO}_2$  or  $\text{cH}$  during hypercapnic hyperventilation is involved in such an intermediary mechanism, modifying the outcome of the chemoreflex signals, cannot be decided from the experiments referred to above, since both  $p\text{CO}_2$  and  $\text{cH}$  exceeded their normal values. However, it should be possible to obtain some information on this point in experiments, in which only one of the two variables deviates significantly from normal levels. The results from such experiments will be presented on p. 63.

The hemo-respiratory reactions following sudden and temporary administrations of  $\text{CO}_2\text{—O}_2$  gas mixtures instead of  $\text{O}_2$  to the chemoceptively intact animal are shown in fig. 4 A and B. By comparing these reactions with those of the chemoceptively denervated animal (fig. 9 A), some further information as to the rôle of the chemoreflex drive during hypercapnic hyperventilation might be obtained. The rise in arterial  $\text{cH}$  and  $p\text{CO}_2$  at the maximum of the hypercapnic hyperventilation is substantially the same in the "intact" and chemoceptively denervated animal, indicating that the increase in alveolar ventilation was approximatively the same. This fact supports the conception that the importance of the chemoreflex component is insignificant in the hyperpnea of high grade hypercapnia.

When, on the other hand,  $O_2$  was suddenly substituted for the  $CO_2$ — $O_2$  gas mixtures, the temporary shift to the alkaline side in the arterial blood reaction became more pronounced in the intact animal, indicating that the alveolar ventilation decreased more slowly, when the chemoreflex drive was in function. This observation is in harmony with the opinion that, as long as the arterial cH and  $pCO_2$  vary within their normal physiological ranges, the arterial  $pCO_2$  stimulates respiration also over the chemoreflex mechanism.

#### b) *Deviations from the Normocarbic Condition.*

The evidence presented above, that the centre responds to changes in cH within itself, and that hyperventilation is set up during acidosis of the hypocarbic type ("metabolic acidosis") in the chemoceptively denervated dog, indicates that the chemoreflexes are not essential for this type of hyperventilation. In "metabolic alkalosis", on the other hand, one would from a teleological point of view expect that during air-breathing the chemoreflex drive should gain increasing importance, remembering that alkalosis is associated with a decreased alveolar ventilation and consequently with a lowered arterial oxygen saturation.

In order to obtain experimental evidence the chemoceptive signals were first blocked in hypercarbic conditions. Fig. 12 shows the effects of temporary withdrawal of the chemoreflex drive under 1) air-breathing, 2) 6.5 %  $CO_2$  in  $O_2$ , and 3) 5.1 %  $CO_2$  in air. The marked increase in arterial cH and  $pCO_2$  under air-breathing indicates that the alveolar ventilation was reduced considerably. This points to an important rôle of the chemoreflex component in the maintenance of the respiratory activity during hypercarbia and air-breathing. When 5.1 %  $CO_2$  was added to the inspired air and hyperventilation was thus set up, blocking of the chemoreflexes revealed, that the chemoreflex support was now definitely less than under air-breathing. It seems highly probable that the greater support of the chemoreflexes in the former case was partly caused by a more marked hypoxic stimulus to the chemoreceptors. Although the arterial oxygen saturation was only somewhat less during air-breathing,

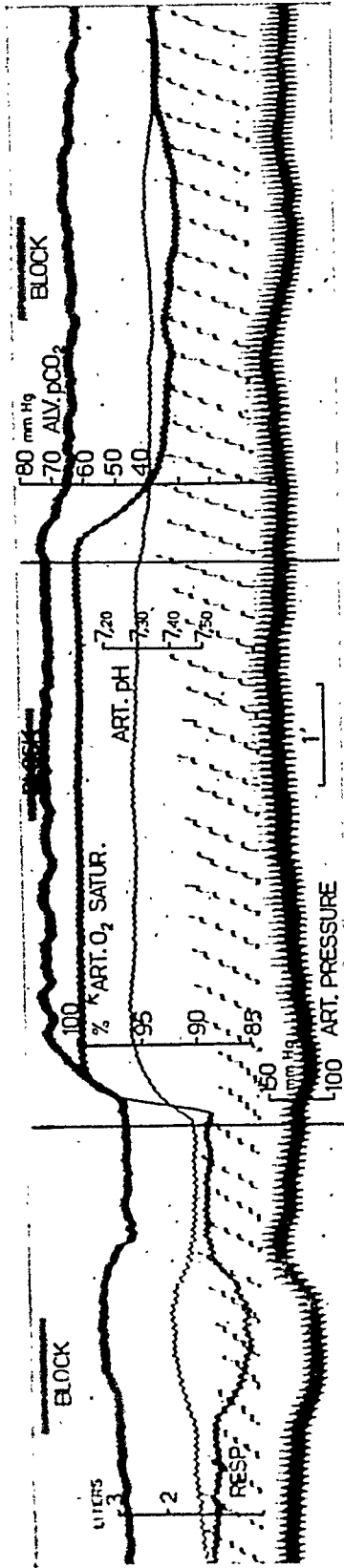


Fig. 12. The figure shows the relative importance of the central and chemoreflex components in the respiratory activity under alkalotic condition during inhalation of air, 6.5 % CO<sub>2</sub> in O<sub>2</sub>, and 5.1 % CO<sub>2</sub> in air. The shifts from one gas to another are marked by vertical lines. During air-breathing blocking of the chemosensory impulses results in a marked decrease of the alveolar ventilation. If 5.1 % CO<sub>2</sub> is added to the inspired air, a very small decrease of ventilation follows the withdrawal of the chemosensory impulses. The difference in blocking reactions is explained mainly by the greater hypoxic stimulation in the former case and also to an exaggerating effect upon the chemoreflex drive exerted by the tissue alkalosis or hypoxia pp. 61—63.

the arterial oxygen pressure was considerably less in this condition. This becomes evident if calculating the  $pO_2$  from the determined pH and oxygen saturation and the standard dissociation curves for oxygen.

It seems to be no reason to assume any central blocking action of  $CO_2$  per se on the signals, which are set up in the chemoreceptors, as suggested by GESELL, LAPIDES & LEVIN. Thus although the arterial  $pCO_2$  during air breathing was considerably higher than normally, the outcome of the chemoreceptor activity was probably not diminished to any significant degree. On the contrary, it seems not impossible, that the chemoreflex drive was actually exaggerated to some extent, be it by a modifying action of tissue alkalosis or hypoxia at the centre or along the chemoreflex nervous pathway. If so, the remarkable difference between the effects of blocking under air-breathing and 5.1 %  $CO_2$  in air will become more intelligible. For under inhalation of 5.1 %  $CO_2$  in air the arterial cH and  $pO_2$  increased to normal levels, and it should therefore be no reason to expect any significant modification of the chemoreceptor influence. Hence, in this case the blocking should reveal the relative activity of the central and peripheral chemosensitive cells.

When the arterial cH and  $pO_2$  were increased to supernormal values by having the dog breathe 6.5 %  $CO_2$  in  $O_2$ , withdrawal of the chemoreflex drive hardly produced any reduction in ventilation (fig. 12). It is therefore evident that the chemoreflexes hardly had any share in the hyperventilation. The peripheral chemoreceptors were without doubt stimulated by increased acidity within themselves, but the chemoreceptor excitation caused only an insignificant part of the respiratory activity. Hence, the response of the centre to the afferent impulses must have been influenced by some intermediary mechanism. The increase in ventilation when the dog was allowed to breathe 5.1 %  $CO_2$  in air, *i. e.* when the firing activity of the peripheral chemoreceptors was not modified to any significant degree, shows that the centre was not firing at its maximum. It therefore seems evident, that the cause of the decreased chemoreflex support under inhalation of 6.5 %  $CO_2$  in  $O_2$  was a modifying action of the increased arterial cH or  $pO_2$  on the



chemoroflexes, either at the centre or along the chemoreflex nervous pathway.

Blocking experiments during hypocarbia and oxygen breathing revealed that only a minor part of the acidotic hyperventilation is maintained by the chemoreflex component. These findings are in harmony with the afore-mentioned observations that the centre itself responds to "metabolic acidosis" with an increased activity. On some occasion, however, it was found that, when the peripheral support was temporarily withdrawn shortly after the injections of HCl, the ventilation decreased more significantly. A possible explanation of the increased chemoreflex support under these conditions would be that the permeability characteristics of the cells within the carotid bodies differ from those of the cells within the centre. Assuming that on HCl-administration the cH within the carotid bodies increased more rapidly, it seems evident that in the stages shortly after the injection the chemoreflex support should be increased.

Additional support for the opinion that the cH changes more rapidly within the carotid bodies than within the centre after intravenous injections of acids was obtained from the following observations. In the chemoceptively intact animal rapid injections of HCl and acetic acid produced a marked increase in ventilation almost at the same moment as the "acidotic" blood passed the chemosensitive regions (fig. 7 C). In animals deprived of their chemoreflex mechanism the same injections produced a slower and lesser increase in ventilation (fig. 7 B). These divergent effects seem to point to a more rapid response of the peripheral chemoreceptors to changes in arterial cH, and furthermore, to an additive action of the central and chemoreflex components in the respiratory defense.

## V. Summary.

A short survey is given of notable conceptions of the respiratory defense against acid-base displacements in the blood. The influence of the discovery of the chemoreflexes upon the validity of earlier theories of chemical control of breathing has been taken into consideration.

The central and chemoreflex components in the respiratory activity during acid base displacements in the blood have been investigated experimentally in dogs under chloralose. Deviations from the normal acid-base balance were produced by administration of  $\text{CO}_2$ — $\text{O}_2$  gas mixtures and intravenous injections of acid and alkaline solutions.

The response of the centre itself to deviations in the acid-base balance was studied in dogs, permanently deprived of all known chemoreflex influence. The interaction of central and chemoreflex drives was followed by observing the hemo-respiratory reactions, which were caused by temporary, repeated withdrawals of the chemoreflex component by means of short-lasting cold-blockings of the chemosensory impulses in the sinus nerves (vagi cut). The hemo-respiratory reactions were judged by the aid of special devices for measuring and recording directly, continuously and simultaneously the pulmonary ventilation, the alveolar  $\text{pCO}_2$ , the arterial pH and the arterial oxygen saturation. The investigations yielded the following data and conclusions.

### *Response of chemoceptively denervated centre (pp. 39—55):*

The centre responds to changes in arterial  $\text{pCO}_2$  and  $\text{cH}$ , but only after a certain time lag, which indicates that the activity of the centre is not directly regulated by the arterial  $\text{pCO}_2$  or  $\text{cH}$ . Evidence is presented that the lack of time-correlation is due to the time required for the centre to come into  $\text{CO}_2$

equilibrium with the blood during its passage through the centre tissue.

The centre responds to "metabolic acidosis" with increased activity and to "metabolic alkalosis" with decreased activity. The buffering alkali content of the centre follows the changes of that in the blood in rather close time-correlation. This conception does not imply similarity in bicarbonate concentration or in changes in bicarbonate concentration. In acidotic conditions the response of the centre to  $\text{CO}_2$  is increased, and conversely, in alkalotic conditions the response is decreased. Among the variables in the acid-base balance —  $\text{cH}$ ,  $\text{CO}_2$  ( $\text{H}_2\text{CO}_3$ ), and  $\text{BHCO}_3$  — the  $\text{cH}$  within the centre tissue is the dominating factor in the control of the self-engendered activity of the centre. Experimental evidence speaks in favour of an *intra-central acidity theory* for the *direct* chemical stimulation of the centre itself. The intracentral  $\text{cH}$  is only to a minor degree dependent on the metabolism of the centre tissue.

*Interaction of central and chemoreflex components (pp. 55—64):*

The chemoreflex component has only a minor share in the hyperpnea of low grade hypercapnia, and does not support the hyperpnea of high grade hypercapnia. Evidence is presented that the outcome of the chemoreflex signals, *i. e.* the chemoreflex drive, is influenced by a modifying action of the arterial  $\text{cH}$  or  $\text{pO}_2$  at the centre or along the chemoreflex nervous pathway.

The respiratory activity is increased during "metabolic acidosis" and decreased during "metabolic alkalosis". The chemoreflex drive gains in importance under alkalosis and eupnea, mainly due to a more marked hypoxic stimulation to the chemoreceptors, and probably to some extent due to an exaggerating effect upon the chemoreflex drive of tissue alkalosis or hypoxia.

Withdrawal of the chemoreflex component during acidotic conditions revealed that the hyperpnea during acidosis is mainly centrogenic.

*Other data and conclusions from the present work:*

The changes in alveolar  $p\text{CO}_2$  and arterial  $c\text{H}$  within the respiratory cycle may become very marked at a slow rate of respiration, especially in alkalotic conditions (p. 36 and fig. 5).

Under physiological conditions the arterial  $p\text{CO}_2$  stimulates respiration also over the chemoreflex mechanism, and consequently, the chemoreflex component is a necessary factor in the finer adjustment of breathing with respect to carbon dioxide as is also the case with respect to arterial oxygen saturation (pp. 56—57 and fig. 10, cf. EULER & LILJESTRAND 1936, 1940).

The peripheral chemoreceptors probably respond more rapidly than do the central chemosensitive cells to changes in arterial  $c\text{H}$  (p. 64 and fig. 7).

## VI. References

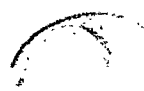
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# APPENDIX





## **A Device for Measuring and Recording Automatically and Continuously the Carbon Dioxide in the Alveolar Air.**

By

C. M. HESSER.

It is well established that the  $p\text{CO}_2$  of the alveolar air is practically the same as in the arterialized blood leaving the lungs. Therefore, determinations of alveolar  $p\text{CO}_2$  will yield a possible method of observing indirectly the  $p\text{CO}_2$  of the systemic arterial blood. Since HALDANE and PRIESTLEY (1905) published their classical method of direct sampling of alveolar air, a number of modifications have been developed for collecting alveolar air in human subjects and animals. (For special reference, see LILJESTRAND 1925).

In studies of the significance of the arterial  $p\text{CO}_2$  for the chemical regulation of the pulmonary ventilation under varying conditions it became necessary to develop a method for continuous recording of this variable. The method, which also involves automatic sampling and analysis of alveolar air, is described and discussed in detail below.

### **Sampling of Alveolar Air.**

In a "continuous" measuring of the  $p\text{CO}_2$  of the alveolar air, *i. e.* the portion of the pulmonary gas actually taking part in the respiratory gas exchange, the variations within the resp-

iratory cycle itself can not be observed because in practice the sampling can only be made from the gas leaving the alveoli during expiration. However, it is mainly the variations in the average alveolar  $p\text{CO}_2$ , that are of physiological significance in the pulmonary ventilation. The  $p\text{CO}_2$  of the gas leaving the alveoli during the expiration will usually at a certain moment correspond to the average alveolar  $p\text{CO}_2$  of the whole respiratory cycle. However, this moment varies with rate and depth of respiration, and too, the  $p\text{CO}_2$  increases during the time necessary for obtaining the sample of alveolar air. On the other hand, if the sample is obtained from a space which still contains some inspired gas at the time of sampling, the  $p\text{CO}_2$  of this sample will have any value between that of the alveolar air and the inspired gas. Therefore, the risk of obtaining a "mixed sample" is increased when the sample is taken early in expiration. The same risk is incurred when the sample is drawn distant to the alveoli and when the respiration is shallow. In order to get proper samples, they must be collected during the last phase of the expiration and/or during the subsequent respiratory pause, and from a space as close to the alveoli as practically possible. In practice the sampling may be made from the trachea in animals or the posterior oral cavity in human subjects.

Several methods for obtaining automatically and "continuously" the alveolar air have been reported, but none seems to satisfy all of the above-mentioned requirements. In the methods, where the last part of each expiration is automatically collected from the proximal end of a tube, attached to the expiratory side of a valve device (HENDERSON and HAGGARD 1925, SMITH and HEINBECKER 1928, BENZINGER and BRAUCH 1934, RAHN, MOHNEY, OTIS and FENN 1946) there will be a considerable risk for getting a "mixed sample". Such a contamination will always be the result, if the volume of expired gas is less than the functional dead space. Moreover, the procedures increase the functional dead space, thereby increasing the average alveolar  $p\text{CO}_2$  and thus disturbing normal breathing. KRAMER and SARRE (1936) described a method for automatic sampling of alveolar air from the trachea of animals. The samples seem to be drawn during the phase, when the velocity or pressure

of the expired gas is highest, i. e. during a short period somewhere in the middle of the expiration, and consequently not during the pause following the expiration as was claimed by the authors. The period of sampling should in many instances be too short to provide a gas analyzer with an adequate amount of alveolar air. Moreover, if the velocity or pressure of the expired gas is very high, the sampling will start almost immediately after the expiration has begun, thus increasing the risk for obtaining a "mixed sample". In the method of LOESCHKE, OPITZ and SCHOEDEL (1939) the samples are drawn from the trachea during the last phase of the expiration and the subsequent respiratory pause. The sampling is accomplished by means of a pump, which is set in function by an electromagnetic device at the moment when a respirometer, inserted beyond the expiratory valve, has reached a certain degree of filling during the expiration. But besides the necessity of having a load on the lid of the respirometer, an arrangement which hampers the expiration, there is also an Hg-contact on the respirometer which needs adjustment every time the depth of expiration changes. These adjustments must be made by an operator and will be difficult or impossible, if the depth of expiration changes rapidly and unexpectedly.

In the present communication the alveolar samples are drawn by a suction pump through a catheter in the trachea of animals, or the posterior oral cavity in human subjects. The principle of the method for automatic sampling of alveolar air is based on the pneumotachygraphical pattern of the pressure (velocity) changes of the tidal air in the upper respiratory tract. The use of the pressure pattern for timing of the suction from the space of sampling seems to be ideal, provided that the suction can be made to begin, when the pressure of the tidal air after passing its maximum approaches zero, i. e. at the end of the expiratory stroke, and to stop, when the pressure becomes negative, i. e. at the onset of inspiration. Because of the constancy of the pressure pattern the timing of the sampling actually remains undisturbed by changes in rate and depth of breathing as well as in resting respiratory level. The pneumotachygraphical control of sampling is effected by the following arrangement (cf. fig. 1). A rubber tube connected to the sampling catheter passes an

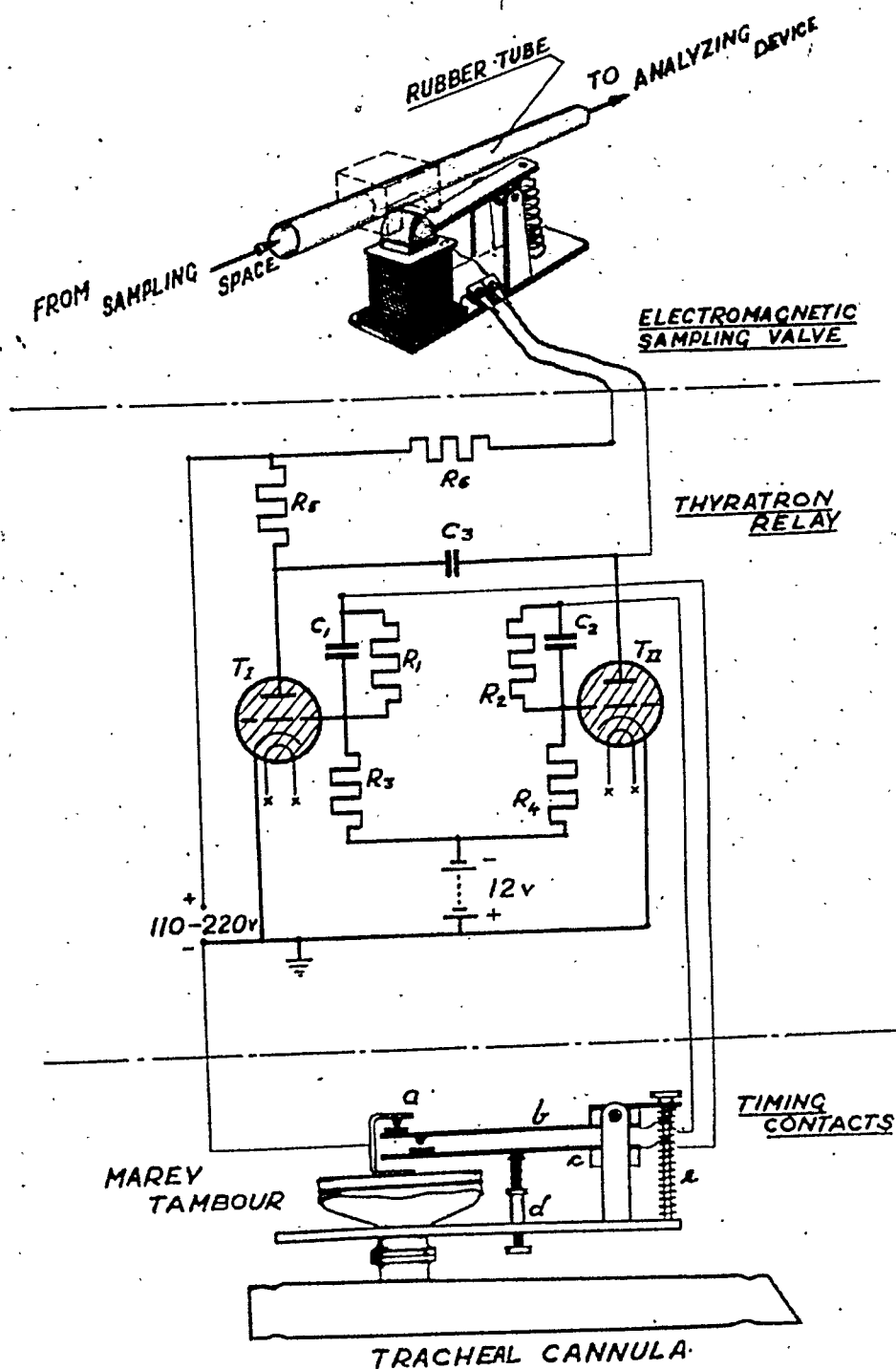


Fig. 1. Schematic representation of device for automatic sampling of alveolar air. For further description see text.  $T_1 = T_{II}$  = Philips EC 50 gas triodes.  $R_1 = R_2 = 1$  megohm.  $R_3 = R_4 = 0.1$  megohm.  $R_5$  and  $R_6$  = load resistances of the anode circuits.  $C_1 = C_2 = 10,000$  pF.  $C_3 = 2 \mu$  F. d and c, insulated screws for adjustment of stationary spring contacts b and c.

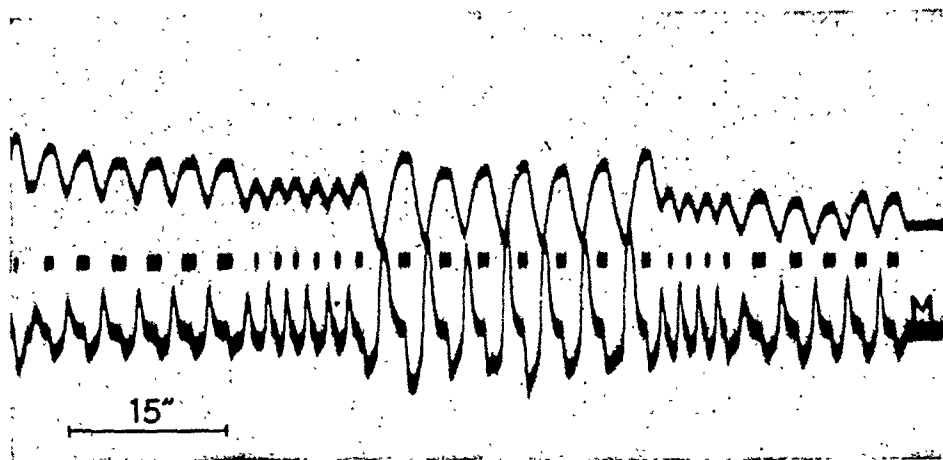


Fig. 2. Automatic timing of sampling, controlled by the pressure fluctuations in the tidal air as expressed by the pneumotachygram. Alveolar air is collected during the last phase of each expiration and subsequent respiratory pause, irrespectively of rate and depth of breathing as well as of resting respiratory level. The periods of sampling are signalled by replacing the electromagnetic valve with an electromagnetic writer (interrupted line). Upper curve = respirometer tracing. Lower curve = pneumotachygram (movement of Marey tambour) as recorded by means of an optical rubber membrane manometer. Expiration  $\uparrow$  in upper tracing. In lower tracing M indicates zero pressure of tidal air. Positive pressures (expiration) above M, negative pressures (inspiration) below M.

electromagnetic device, which compresses the tube, when not operated. Power to the electromagnet is supplied by a double thyatron relay, which includes two Philip EC 50 gas triodes. The Philip EC 50 gas triode permits a high discharge current, sufficient to operate the electromagnetic valve. This valve is inserted into the anode circuit of one of the thyatrons. The relay is controlled by the special series of electrical contact-making of a modified Marey tambour. The tambour is provided with the contacts *a*, *b* and *c*, and is attached to a mouthpiece or a breathing mask, or in the case of animals to a side-opening in a tracheal cannula. Under the influence of the varying pressure of the tidal air during the respiratory cycle, the rubber membrane of the tambour moves out and in, giving graphically the pneumotachygram (see fig. 2).

During a respiratory cycle the apparatus functions in the following manner. When during the last phase of the expiration the contact *a* of the rubber membrane first touches the stationary spring contact *b*, the capacitor  $C_2$  makes contact with

the cathodes. This induces a momentary reduction of the negative control grid bias of the thyatron II ( $T_{II}$ ), which causes the tube to fire. The electromagnet is then thrown in gear, thus allowing sampling to take place. When the membrane also contacts the second stationary spring contact  $c$  at the onset of the inspiration, the negative grid bias of the thyatron I ( $T_I$ ) is reduced momentarily in the same way. Hence, this tube will fire. However, owing to the presence of the capacitor  $C_3$  between the anodes, the firing of one tube will always cause the other to be extinguished, and therefore the sampling is now interrupted by the release of the electromagnet. Thyatron II remains non-conducting until the moment its grid bias is again reduced momentarily at the end of the next expiration. In this way sampling will occur only during the last phase of the expiration and during the subsequent respiratory pause (fig. 2). This means that irrespectively of rate and depth of breathing, the period of sampling from the upper respiratory tract permits the securing of a gas sample, the  $pCO_2$  of which approximates that of the average alveolar air.

Additional features of the device are the rapid and precise action of the electromagnetic valve. Also, the exceedingly small currents passing the contacts of the tambour leave the contacts clean by excluding arc formation.

### **Continuous and Automatic Recording of the Carbon Dioxide in the Alveolar Air.**

Several physical methods for determination of  $CO_2$  in samples of alveolar air have been developed, most of them based on the specificity of the refractive index or of the thermal conductivity of the gas. Optical interferometric gas analysis seems not to be suitable for continuous and automatic recording of the alveolar  $pCO_2$  (see BENZINGER and KITZINGER 1948). For this purpose the hot wire method of measuring the thermal conductivity of the gas appears to be the simplest and most generally employed. The principles and theory of the method have been reviewed i. a. by DAYNES (1933) and REIN (1937).

In continuous recording of the alveolar  $pCO_2$  with the hot wire method of REIN (1937), the intermittently obtained samples

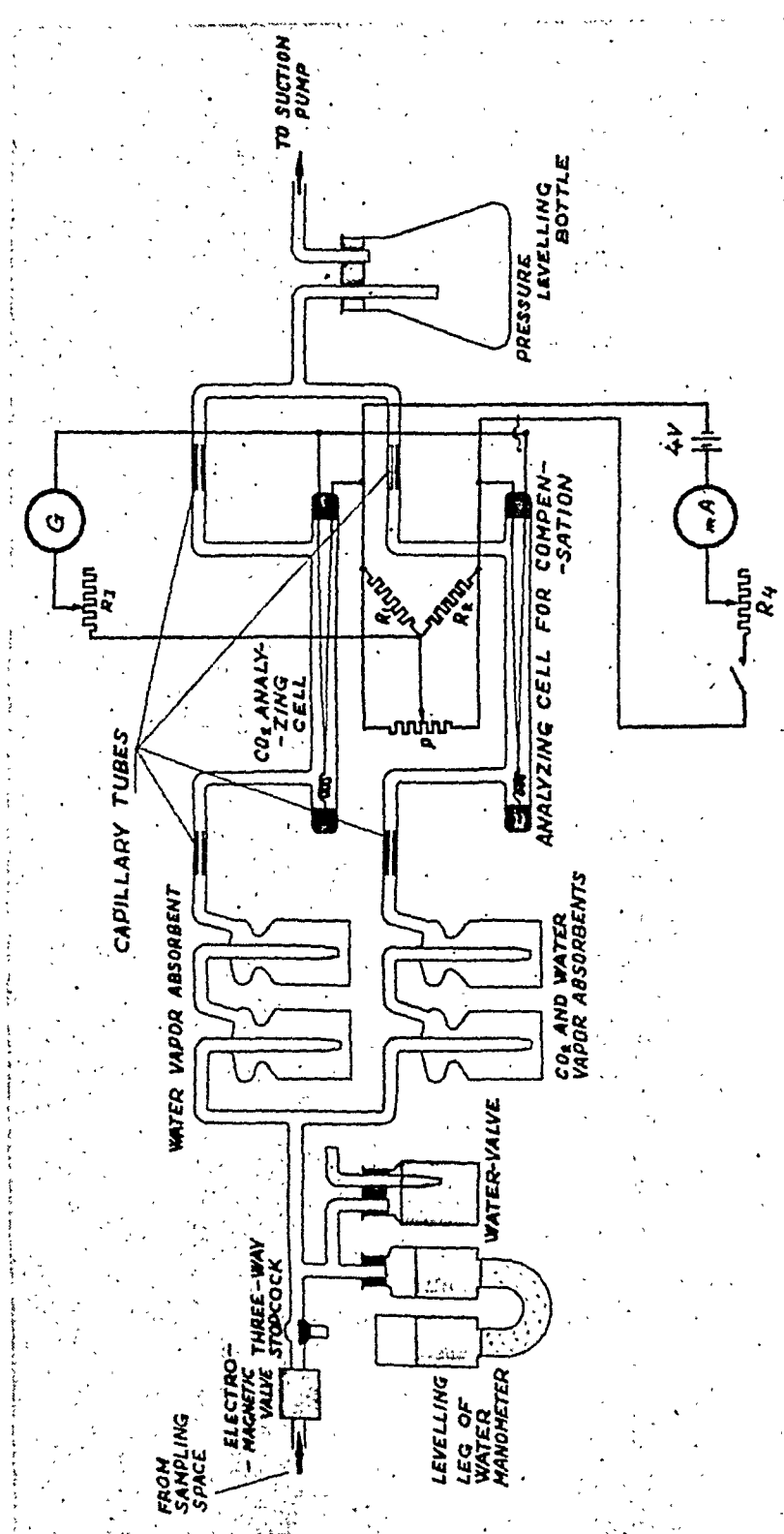


Fig. 3. Schematic representation of CO<sub>2</sub>-analyzer for intermittently drawn alveolar samples.  
For further description see text.



of alveolar air must pass the analyzing cells at a constant rate. This is attained by causing the samples to stream out in an open tube, from which the gas to the analyzer is continuously sucked. However, in this arrangement the samples of alveolar air must be 6—10 times larger than the gas actually passing the analyzer. Moreover, another objection is that the analyzing cells of Rein are fairly difficult to manufacture. On the other hand BERG (1947) has described an easily built thermal conductivity gas analyzer. However, Berg's analyzer can not be used for automatic recording of the alveolar  $p\text{CO}_2$ .

The following is a brief description of a thermal conductivity gas analyzer, especially designed for recording continuously and automatically the  $p\text{CO}_2$  of intermittently obtained samples of alveolar air (cf. fig. 3).

The arrangement for insuring a continuous and steady flow of alveolar air through the  $\text{CO}_2$ -analyzer is as follows: Between the electromagnetic sampling valve and the suction pump a water-manometer and the analyzing device are connected in series. As long as the valve remains open, the pump will suck gas through the analyzer directly from the original space of sampling. Meanwhile the water level in the manometer falls and a fraction of the alveolar air is stored above the water. When the valve closes, the stored gas is drawn through the analyzer, thus preventing interruption of the flow in the analyzer. The rhythmical fluctuations of pressure deriving from the manometer and the pump are prevented from being transmitted to the analyzing cells by means of narrow capillary tubes on both sides of the cells. For the same purpose a pressure levelling bottle is placed before the pump.

To insure the analyzer of a sufficient amount of gas when the valve is closed, the maximum volume of the gas above the water in the manometer can be adjusted by elevating or lowering a levelling leg of the manometer. A safety water valve prevents the water of the manometer from entering the analyzer in case the intermittent obtaining of gas samples is interrupted for longer periods. A supply of atmospheric air or known test mixtures of  $\text{CO}_2$  for checking zero reading or empirical calibration of the analyzer may be made by means of a three-way stopcock placed after the electromagnetic valve.

After the gas has been divided by a T-tube, one stream is drawn through two bottles, one with 33 % KOH and the other with concentrated  $\text{H}_2\text{SO}_4$ . These act as absorbents for  $\text{CO}_2$  and water vapor. The gas then enters an analyzing cell. The other gas stream is drawn through two bottles with concentrated  $\text{H}_2\text{SO}_4$  and then through another analyzing cell. In order to enlarge the contact surface between the gases and the absorbents, the absorbent bottles also contain small glass prisms. The spaces on top of the fluids in the bottles are of approximatively the same size and should be kept as small as possible. The analyzing cells and the tube-connections should also be kept at a minimum. All this will ensure a rapid and matching flushing out time in the two lines.

Each one of the analyzing cells consists of a thin, doubled, and electrically heated platinum wire inside a glass tube with a T-connection at each end. The wire is held taut with a helical spring between the angled end of the wire and a Bakelite plug at one end of the tube. The two free ends of the wire are soldered on copper filaments passing through a Bakelite plug at the other end of the glass tube. The plugs are sealed into place with De Khotinsky cement. The platinum wires of the two cells constitute one pair of the resistance-arms in a Wheatstone bridge arrangement, whereas the other pair is formed by two constantan filaments  $R_1$  and  $R_2$  with matching resistances. The two cells and the resistors are immersed in a bath of liquid paraffin in order to prevent transient changes in the heat uptake of the environment and subsequent alterations of the resistances. The current flow from a storage battery through the Wheatstone bridge is controlled by a rheostat  $R_4$  and a milliammeter  $mA$ , connected in series. The changes in resistance of the platinum wires are recorded on photographic paper by means of a sensitive mirror galvanometer  $G$  in the Wheatstone bridge. For adjusting the electrical zero and the sensitivity of the bridge a potentiometer  $P$  and a rheostat  $R_3$  are connected to the bridge as shown in the figure.

The arrangement of the assembly has the advantage that changes in the thermal conductivity of the analyzed gas, which refer to gases other than  $\text{CO}_2$ , will have negligible influence on the galvanometer readings. The deflections of the galvano-

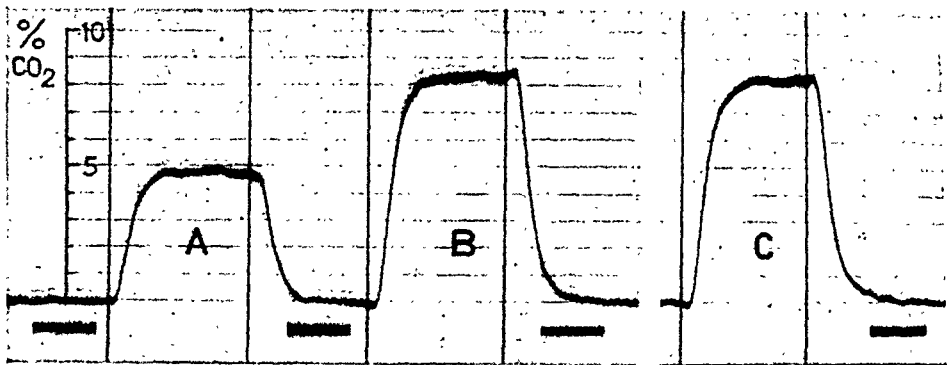


Fig. 4. Time composition and empirical calibration curves of the  $\text{CO}_2$ -analyzer. Between vertical lines known test mixtures of  $\text{CO}_2$  (analyzed by the Haldane volumetric method) are supplied via the sampling catheter, the electromagnetic valve being open. Latency time and half time of the adjustment period amounts to about 6 seconds each. Readings reflect almost linearly the percentage of  $\text{CO}_2$ , gases other than  $\text{CO}_2$  having negligible influence. Note also constancy of zero-line and of amplitude of deflection for a gas of fixed composition. Time marking = 30 seconds.

A. 4.86 %  $\text{CO}_2$  in  $\text{N}_2$ . — B. 8.87 %  $\text{CO}_2$  in  $\text{O}_2$ . — C. As in B., after continuous operation of analyzer for two hours.

meter therefore reflect practically linearly the  $\text{CO}_2$  percentage of the dried gas samples (see fig. 4).

Since the gas needs a certain amount of time to pass through the entire analyzing assembly, the reading lags behind the event as is also the case with other similar hot wire methods for gas-analysis. At a flow of about 1 c. c. per second through each cell the latency time is about 6 seconds, i. e. the time elapsing from the moment the gas is drawn from the space of sampling until a fraction of the gas reaches the cells. The half-time of the subsequent "adjustment period", i. e. the time required for 50 % flushing out to constant conditions in the cells (50 % of full galvanometer deflection), also amounts to about 6 seconds. Thus the beginning of a galvanometer deflection always indicates a change in the alveolar  $\text{pCO}_2$ , which has taken place 6 seconds earlier. At every moment the amplitude of the reading reflects the average  $\text{CO}_2$  percentage of a series of dry alveolar samples, drawn during a period of between 12 and 20 seconds previous to the beginning of the latency time.

An example of the constancy of the zero line is also shown in fig. 4. After continuous operation of the analyzer up to 16

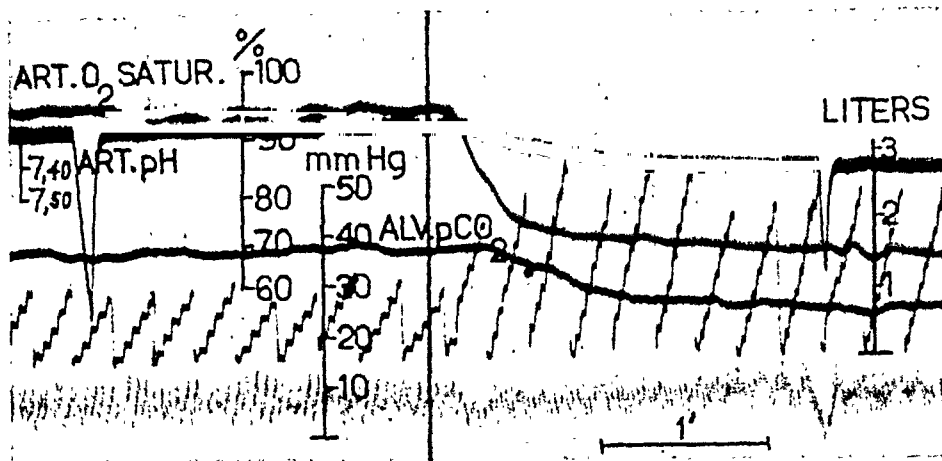


Fig. 5. Dog 22 kg., chloralose. Automatic and continuous measuring and recording of average alveolar  $p\text{CO}_2$  by method described in text. Shift from room air to 10 %  $\text{O}_2$  in  $\text{N}_2$  at marking. Note decrease of alveolar  $p\text{CO}_2$ , arterial cH and oxygen saturation during hypoxic hyperventilation. Dips of pH-tracing indicate interruption of recording for calibration.

*Legend:* Arterial pH, recorded by a glass electrode arrangement. Arterial oxygen saturation, recorded by a photoelectric barrier-layer cell oximeter. Both measured in a femoral. sin. Height of stair tracing shows volume of expired gas after every 15 seconds period. Lowest curve = systemic arterial blood pressure (approximately 140 mm. Hg).

hours, the drift of the zero line has been proved less than corresponding to 0.1 %  $\text{CO}_2$ .

Fig. 5 illustrates the combined function of sampler and analyzer in an experiment on a dog under chloralose.

### Summary.

A short critical review is given of a number of earlier methods for automatic sampling of alveolar air.

A description is given of an arrangement, especially designed for continuous and automatic recording of alveolar  $p\text{CO}_2$ , and consisting of the following devices:

- 1) An electronic sampler device, controlled by the pressure fluctuations in the tidal air as expressed by the pneumotachygram. The samples are collected via a narrow catheter from the trachea of animals or in human subjects the posterior oral cavity. The samples are drawn automatically during the last phase of each expiration and the subsequent respiratory pause,

irrespectively of rate and depth of breathing as well as of resting respiratory level.

2) An easily built and accurate thermal conductivity analyzer for recording continuously the  $p\text{CO}_2$  of intermittently obtained samples. The analyzer is especially designed for shortness of time lag and automatic compensation of disturbances from co-existent gases, as well as for suppression of zero drift.

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